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Oral dose toxicity vs. tissue residue levels of arsenic in the honey bee (Apis mellifera L.)

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ORAL DOSE TOXICITY VS TISSUE RESIDUE LEVELS OF ARSENIC IN THE HONEY BEE (*APIS MELLIFERA* L.)

By

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Oral Dose Toxicity vs Tissue Residue Levels of Arsenic in the Honey Bee (Apis mellifera L.)

Director: Clarence C. Gordon

The purposes of this study were to supply new information on the median lethal dose of arsenic to honey bees and to compare oral dose toxicity and tissue residue levels of arsenic.

Honey bees were orally dosed for 24 hours with arsenic trioxide (As_2O_3) and sodium arsenite ($NaAsO_2$). Dead bees were collected each day for 6-7 days and a record kept of the number of dying bees and bee behavior. All honey bee samples were kept separated and stored in a freezer until analyzed.

Prior to analysis, whole bees were digested by a perchloric, sulfuric and nitric acid digestion. Samples were analyzed on the atomic absorption spectrophotometer using the arsine generation method. Additional analytical tests were conducted to study an unknown residue in the digested samples, the stability of the arsenic and sugar solutions and the accuracy of the chemical analysis procedure. Statistical analysis was conducted utilizing the Statistical Package for the Social Sciences. The Miller and Tainter probit method was employed to calculate the median lethal dose values.

The results suggest a colony influence on the susceptibility of bees to arsenic. Colonies 1 & 3 and 2 & 4 had similar LD_{50} values for arsenic trioxide while colonies 1 & 2 had close values for sodium arsenite. Colony LD_{50} values ranged from 1.52 to 3.04 ug/bee of elemental As for arsenic trioxide and 0.330 to 0.540 ug/bee of elemental As for sodium arsenite. The aggregate LD_{50} values were 2.30 ug/bee of elemental As for arsenic trioxide and 0.544 ug/bee of elemental As for sodium arsenite. Regardless of colony, for each given dose level, bees had similar amounts of arsenic in their bodies and a common response to the arsenic. Thus it appears that colonies of honey bees differ in susceptibility to arsenic but not in their general response and accumulation of the poison. The statistical results verify that the arsenic dose level was the main influencing factor throughout the tests.

A very strong positive correlation was found between the calculated dose administered to the honey bees and the resultant tissue residue of arsenic. Tissue residue levels were greater than the oral dose. Confirmation studies are necessary to determine whether this relationship holds for field situations.

Acknowledgements

This thesis research would not have been possible without the tremendous support and assistance from many people. I am indebted to them all.

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CHAPTER I

INTRODUCTION

Beekeeping is an important agricultural industry in Montana and in the United States. Within Montana alone, the value of honey bees as producers of honey and beeswax annually approximates $4.3 million wholesale, while the United States honey and beeswax production annually amounts to about $100 million wholesale (estimates based on Montana Agricultural Statistics for 1976 to 1977). However, the value of honey bees as pollinators of agricultural crops, ornamentals, and wild plants far exceeds that of the honey and wax. In fact, the pollination service rendered by honey bees to agricultural crops alone appears to be 20 to 100 times more valuable than the honey and wax produced (Winski, 1974; McGregor, 1976). The dollar contribution to ornamentals and wild plants is inestimable, but obviously great.

In the late 1800's man became increasingly aware of the damaging effects of his activities to beneficial insects. At this time arsenic-based insecticides were beginning to be used. Extensive beekills from airplane dusting with calcium arsenate, Paris Green, and other arsenical insecticides occurred throughout the 1930's and 40's in California, Utah, Arizona and Texas confirming the high sensitivity of honey bees to arsenic. In addition reports of damage to bees from industrial sources began to appear. These reports documented beekills from arsenic air pollution in Czechoslovakia, Germany, France and Luxembourg. Laboratory and field investigations confirmed the toxicity of arsenic to honey bees, established the primary mode of

action and the influence of various environmental factors. Bees also were shown to be biological accumulators of arsenic.

Today the sensitivity of honey bees to pesticides and pollution is well documented. Laboratory and field studies tend to emphasize the sensitivity of honey bees to organic insecticides; yet, there are still occasional beekills from inorganic insecticides such as the use of calcium arsenate (Wood and Wood, 1962). Furthermore there is continuing concern in some geographical areas with industrial poisoning of honey bees. One such area is the Anaconda-Whitehall-Deer Lodge region of Montana. This region has had a history of beekills and loss of colony viability. Much of this is attributed by the local beekeepers to arsenic air pollution from the copper smelters of the region. The problem of arsenic poisoning seems to be continuing in this area with possible arsenic caused beekills being recorded at Whitehall during the summers of 1974 and 1978.

Honey bees also are known to be highly susceptible to harm from other sources of pollution. There is a growing movement to utilize honey bees as biological monitors. Plans for research which will determine more precisely the role of honey bees as biological monitors are now being formed (Luepke, 1978; Bromenshenk and States, 1980).

Rationale

The purpose and rationale of this study was two fold. First, there is a lack of recent research concerning honey bees, pollution and inorganic poisons such as arsenic. The majority of studies were done during the early 1900's when many of the insecticides were arsenic based. Even at this time there were only a few investigations

concerned with arsenic as an air pollutant. One intent of this study was to supply new information on the toxicity of arsenic to honey bees.

Second, two methods have been used to describe the toxicity of poisons to honey bees. These are the determination of median lethal dose via oral dose tests and tissue residue analysis of poisoned bees. Oral dose tests are conducted in the laboratory to determine toxicity; while tissue residue levels are utilized in the investigation of beekills in the field. Neither have been compared to the other.

Thus beekeepers, researchers and lawyers are unable to reliably relate laboratory test data (median lethal dose) to field data (tissue residue). Questions have arisen as to whether there is any correlation between these methods and if so what kind. In this study both methods were used on the same set of honey bees. Oral dose tests were conducted, the resulting bee samples analyzed for tissue residue of arsenic and a statistical analysis of the data was used to determine the correlation between the arsenic dose administered and the resultant tissue residue.

CHAPTER II

LITERATURE REVIEW

Physical Aspects of Arsenic

All arsenic insecticides are derived from white arsenite, the name for commercial arsenic trioxide, As_2O_3. This white solid is obtained from the flue dust of smelters using arsenic bearing ores; i.e., those for copper, lead, iron, silver and gold. In 1930, 90% of the white arsenite came from copper smelters in Montana and Utah.

There are two major oxide forms of arsenic: arsenic trioxide and arsenic pentoxide. From these are derived two series of salts, the arsenites and arsenates, which differ in their toxicity and uses as insecticides. Of the arsenites, the most important compound was Paris Green, the aceto-arsenite of copper. This compound was widely used and is well known, being first made as early as 1814. Paris Green has a very high arsenic content. It is fairly unstable, being easily broken down into soluble arsenic by water percolation. Soluble arsenic burns the foliage of plants and is poisonous to beneficial insects. Despite drawbacks, Paris Green was used for spraying potatoes and apple trees, as insect baits and as a spray on water for malaria. In general the arsenites are more active then the arsenates and are more unstable and soluble. (Shepard, 1939).

Notice, it is the arsenites which are derived from smelters. Thus, any arsenic present as a pollutant in smelter gases will tend to be in the most toxic and active form. This assumes that arsenite is the main form released.

Because arsenates are more stable than arsenites, they offered advantages for development as insecticides. The two main forms used were acid lead arsenate and calcium arsenate. Acid lead arsenate was first made in 1892 and was used principally for codling moth control in apple orchards. Calcium arsenate is more injurous to plants and was used on hardier crops such as potatoes, cotton, and timber. (Shepard, 1939).

Factors that Effect Toxicity

Arsenicals are stomach and protoplasmic poisons. When consumed by the insect, they are absorbed through the mid-gut or ventriculus wall. A reaction with the cellular protoplasm of the epithelium then occurs, often precipitating protein and inhibiting other functions. Also some arsenicals such as sodium arsenite are limited contact poisons. As such, they are absorbed through the cuticle or sensillae of the insect. (Brown, 1951).

There are numerous factors which influence the toxicity of arsenic. As mentioned, the chemical form of the compound is very important as is the formulation of the compound. Arsenicals have been used as dusts, sprays and baits. Fine dusts are considered the most hazardous. These dusts are more prone to drifting (Eckart, 1944), can be more easily collected with pollen, and are more soluble (Shepard, 1939). Solubility is another main factor since the rate of penetration of the poison into the insect body and its elimination affects toxicity. Temperature, humidity, the weather, carbon dioxide, and the particle size of the compound can affect solubility. Calcium arsenate, acid lead arsenate and copper arsenate are more toxic at

lower temperatures. At low termperatures, the metabolism of the insect is decreased, making it more difficult to cope with the effect of these poisons. Although less poison is necessary, it takes longer to kill the insect. On the other hand, higher temperatures increase metabolism and activity which will enhance the biochemical action of the poison. (Ellisor and Blair, 1940).

Humidity and weather factors such as fog and dew increase the solubility of arsenic and promotes its decomposition. In addition liquid sprays may not evaporate as rapidly on cold, wet days, thus exposing water seeking bees to them. This influence has been demonstrated by the higher occurrence of beekills on colder, wetter days (Milum, 1930). Carbon dioxide has also been found to produce large amounts of soluble arsenic in suspensions of Paris Green, zinc arsenite and calcium arsenate (Shepard, 1939). Therefore colonies located close to roads or other sources of high carbon dioxide may be more susceptible to the above insecticides.

An oral dose study on the toxicity of acid lead arsenate, calcium arsenate, phenothiazine and cryolite to honey bees (Bertholf and Pilson, 1941), demonstrated that particle size has a significant affect on toxicity. In general, fine particles are more toxic then coarse, each size having a different lethal dose. The overall median lethal dose of calcium arsenate and arsenic pentoxide were 0.6 ug/bee of elemental As and for lead arsenate 13.0 ug/bee. When tested by particle size, the median lethal dose of calcium arsenate was 0.7 ug/bee of elemental As for fine and medium particles, 0.6 ug/bee for commercial and 1.3 ug/bee for coarse particles. Acid lead arsenate had

median lethal doses of 5.0 ug/bee of elemental As for fine, 13.0 ug/bee for commercial and 185.0 ug/bee for coarse particles. Similar results have been obtained for Paris Green and lead arsenate in tests with Epilachna, phytophagous Ladybird beetles, and honey bees (Brown, 1951).

The secretions and pH of the honey bee stomach and intestines affect the solubility and absorption of arsenic. Tietz (1924) demonstrated that the digestive secretions of the stomach increase the solubility of arsenate of lead by 1.23 times in comparison to water alone. The intestinal fluids were even more effective, increasing solubility 3.75 times. On the other hand, the pH of the ventriculus or mid-gut has been found to have a buffering action on sodium meta-arsenite. The minimum lethal dose was found in this study to be between 0.1 to 0.2 ug of elemental As/bee. Two buffering systems are at work, a phosphate system and one that was unknown as of 1934. It is hypothesized that the irritation of the ingested arsenic stimulates an extraordinary secretion of buffering substances (Hoskins and Harrison, 1934).

The pH of the honey bee ventriculus also influences susceptibility to different compounds. In the above study concerning the effect of particle size, it was found that calcium arsenate was much more toxic then lead arsenate. It was felt that the solubility of calcium arsenate is enhanced by an acid condition while lead arsenate requires a basic environment. The ventriculus of the honey bee is slightly acid with a pH of 6.3 (Hoskins and Harrison, 1934).

Certain body structures of the honey bee can increase

susceptibility to contact poisoning by arsenic. For example contact of the antennae of locusts by sodium arsenite rapidly produces death. Other quick entry points are the wings and tarsal chemoreceptors. (Brown, 1951).

Other factors which influence toxicity are health and nourishment. Honey bees are very susceptible during the early spring months before the colony has recuperated from the winter (Bromenshenk, 1978). Poisoning at this time, will greatly deplete the work force and nurse bees, both of which are necessary for the buildup of the colony and brood. Furthermore chronic exposure to arsenic may weaken a colony. Such a colony will be more prone to disease and winter die off (Toshkov, et al, 1974). Added stress such as a food shortage or sudden cold spell could trigger a sudden increase in mortality (Bromenshenk, 1978; Knowlton, et al, 1950).

Reports of abnormal defecation by honey bees affected by arsenic poisoning indicate a possible elimination mechanism. The ability to eliminate a poison would decrease the speed by which it is absorbed and thus its toxicity. Hoskins and Harrison (1934) observed defecation when determining the buffering power of the honey bee stomach. In another case, bees poisoned in the field had distended abdomens which exuded a viscous mass of golden yellow feces. Considerable spotting of the combs within affected hives was also observed (Milum , 1930). In addition I have heard of this occurring in the Deer Lodge Valley of Montana (personal communication from Jerry Bromenshenk).

Honey bees confined in cages were not observed to void any of

the arsenic consumed (Cook and McIndoo, 1923). Such an observation is reasonable since honey bees usually defecate only during flight. Thus if defecation does occur either in the hive or in cage tests, it may indicate tremendous trauma.

Histological and Physiological Effects of Arsenic to Honey Bees

The toxicity of arsenic is generally attributed to tissue and epithelium disintegration and protein precipitation. In addition arsenic has been found to affect the haemocytes of insects by decreasing the haemocyte count, stimulating the disintegration of these cells, and changing the chemical composition.

One physical effect of arsenic is to reduce intracellular respiration by uncoupling oxidative-phosphorylation (Bromenshenk and States, 1980). For instance the arsenite form of arsenic disrupts the oxidative decarboxylation of pyruvic acid in the breakdown of carbohydrates. Arsenate does not have the same effect, although it is believed that many arsenate poisons are reduced to arsenite, which increases toxicity (Brown, 1951). More recently, arsenite has been found to inhibit cholinesterase activity. Many organic phosphate insecticides also affect honey bees in this manner. Thus if bees have been exposed to other insecticides, they could be even more susceptible to arsenite poisoning. (personal communication from Yolanda Lehner, USDA SEA AR Bee Research Unit, University of Wisconsin, Madison, Wisconsin).

Honey bees collect arsenic while foraging for nectar, pollen or water. Lethal amounts of arsenic have been found in pollen samples and plant blossoms from areas of intense smelter activity or

agricultural spraying (Knowlton, et al, 1950). If the poison is ingested immediately, the bee may die before reaching the hive. Often contaminated pollen is carried to the hive and stored. It remains toxic for months, killing brood and the nurse bees which ingest pollen in preparation for feeding it to the brood.

Arsenic poisoned honey bees display symptoms such as loss of flight, distended abdomens, and diarrhea. At times, poisoned bees can be seen attempting to leave the hive. They take off at a run, fly short distances, and end up hopping and crawling (Eckert and Allinger, 1935). On cold days, the area surrounding the hives will be covered with crawling or dead bees (Milum,1930). In cage tests of arsenic poisoning, honey bees become very inactive, and stop eating. Their abdomens become swollen and they cannot fly but stagger around dragging their abdomens. Less defecation is observed then that of field poisoned bees. (McIndoo and Demuth, 1926).

Arsenic poisoning of a honey bee colony can have drastic ramifications. Honey bees foraging for nectar and water will often die of poisoning in the process of foraging in the field. The loss of these honey bees can be especially important during hot weather. At such a time, the loss of bees to collect water can be detrimental, eliminating the ability of the honey bees to properly cool the hive through evaporation.

The majority of the honey bees killed in the hive are nurse bees. They are reported to leave the hive when poisoned, before feeding the poison to the brood. The poison source for nonforaging adult bees is contaminated pollen, nectar, and/or water brought in by the

foragers. Unsealed brood are killed by being fed contaminated food, the bees often dying in their cells either just before or at the time of emergence. In badly affected hives, black mummies of pupae are found. Those larvae that remain, die of exposure and starvation due to neglect and the death of the nurse bees. The queen is usually the last bee to be affected, if at all. In instances of severe poisoning, she may leave the hive with only a handful of bees (personal communication from Mr. Ballantine, owner of Cloverdale Apiaries, Manhattan, Montana). Thus in a badly poisoned hive the whole colony can be killed either directly or indirectly (Eckert and Allinger, 1935).

Toxic Levels of Arsenic to Honey Bees

One of the earliest reports of arsenic poisoning of honey bees was in 1881 by G.M. Thompson, who observed the death of bees from Paris Green sprayed on a blooming pear tree. Similar reports continued to be cited throughout the late 1800's and early 1900's (Shaw, 1941). It was not until 1918 that formal research or reports on the lethal dose of arsenic were made. At this time both Troop (1918) and Price conducted laboratory and field studies, citing 0.5 ug/bee arsenic trioxide as a lethal dose (Shaw, 1941).

Before 1920 the majority of bee poisonings and studies were involved with the spraying of fruit trees with arsenical insecticides. In the 1930's airplane dusting of crops emerged as the prominent problem. Eckert and Allinger (1935, 1936) observed the behavior of and damage to dusted colonies. During the first year up to 150 hives were lost. The next year at least 22 hives were killed outright and

the remaining reduced by 50% with the death of all unsealed larvae. Often these hives did not revive enough to outlast the winter.

Table 1 lists the arsenic dose levels reported in the literature from laboratory dose tests and from samples collected from the field and analyzed for tissue residues of arsenic. These values vary depending on the chemical compound, particle size, and mode of contact. In general the median lethal dose for elemental arsenic appears to be within the range of 0.2 to 0.5 ug/bee.

Industrial Poisoning of Honey Bees

Root (1907, 1941) reported extensive damage and litigation with smelters in Utah and Texas. In the Salt Lake Valley, Utah beekeepers experienced a drop from ten thousand colonies down to no more then ten over a ten year period. These beekeepers obtained an out of court settlement of $60,000 in 1907. (Root, 1907).

The problem in Utah continued throughout the early 1900's. From 1870 to 1908 20 or more copper and lead smelters were in operation in the Salt Lake Valley. In 1907 almost all the smelters were closed due to litigation from the farmers. In 1908 a few were allowed to reopen. Since 1908 only three smelters have been in operation, yet beekills continued (Knowlton, et al, 1950).

Several surveys of soil samples about the state indicated excessive amounts of arsenic in smelter areas and sprayed orchards (Knowlton, et al, 1948). The pattern of beekills correlated with smelter areas and to some extent the period of smelter activity. Data supported the conclusion that the majority of adult honey bee losses in the Salt Lake County area was caused by arsenic-containing

TABLE 1

ARSENIC DOSE LEVELS REPORTED IN THE LITERATURE

Author	Date	Levels Reported	Comments
Troop,J.	1918	0.5 ug/bee	As_2O_3, lethal dose
Cook,F.C. N.E. McIndoo	1923	0.5 ug/bee	Metallic As, lethal dose
McIndoo,N.E. G.S. Demieth	1926	0.4-0.5 ug/bee	Elemental As?, lethal dose
Hoskins,W.M. A.S. Harrison	1934	0.1-0.2 ug/bee	Elemental As, lethal dose
Eckert,J.E. H.W. Allinger	1935	140 ppm	Dead bees, $Ca_3(AsO_4)_2$
		13-39 ppm	Crawling bees
		27-28 ppm	Washed pollen bearers
Bertholf,L.M. J.E. Pilson	1941	0.6 ug/bee	Calcium arsenate
		0.7 ug/bee	fine & medium particles
		0.6 ug/bee	commercial size particles
		1.3 ug/bee	coarse particles
		13.0 ug/bee	Acid lead arsenate
		5.0 ug/bee	fine particles
		13.0 ug/bee	commercial particles
		185.0 ug/bee	coarse particles
Sturtevant,A.P. et al	1941	0.023,0.0044,0.006 ug/bee	Suspected poisoning
		0.00005,0.00014 mg/bee	Controls
Beard,R.L.	1949	0.8 ug/bee	Body cavity injection
		0.0046 ug/bee	External feeding
			Sodium meta-arsenite elemental As
Knowlton,G.F. A.P. Sturtevant C.J. Sorenson	1950	0.0-0.15 ug/bee	Normal
		0.16-0.20 ug/bee	Possibly harmless
		0.21-0.29 ug/bee	Shortened life
		0.30 + ug/bee	Lethal dose F.E. Todd Table
Rousseau,par(M.) Mme Pangaud	1959	0.4-0.5 ug/bee	Elemental As, lethal dose
Wood,G.W. F.A. Wood	1962	1.4-2.1 ug/bee	Elemental As Lethal dose to bumble bee Calcium arsenate
Anderson,L.D. E.L. Atkins	1968	0.0-1.99 ug/bee of toxicant	LD_{50} range - Group 1: Highly Toxic Pesticides
Lillie,R.J.	1972	0.5 ug/bee	Elemental As, lethal dose
Atkins,E.L.[1]	1975		Dusting tests
		30-242 ug/bee	Arsenic trioxide
		75 ug/bee (1954)	Calcium arsenate
		24 ug/bee (1969)	Monosodium acid methanearsonate

TABLE 1

Author	Date	Levels Reported	Comments
		218 ug/bee (1969)	Disodium methanearsonate nontoxic
		157 ug/bee (1969)	Dimethyl arsenic acid nontoxic
		6 ug/bee	Arseomethane As-1,2-disulphide toxic
			Oral dose tests, LD_{50}
		0.072,0.072 ug/bee	Arsenic trioxide, acute dose
		0.104,0.070 ug/bee	Arsenic trioxide, chronic dose
Montana Dept.of Health and the Environment Chemistry Laboratory Bureau[2]	1974	0.207-0.25 ug/bee	La Velle, dead bees
		0.316 ug/bee	La Velle, live bees
		0.112 ug/bee	Wise River, dead bees
		0.045 ug/bee	Bohern, dead bees
		0.024 ug/bee	Three Forks, dead bees
		0.031 ug/bee	Siebing Ranch,Helena,dead bees
Bromenshenk, J.J.	1978	0.008-0.024 ug/bee	Live bees,pristine area
Industrial Poisoning of Honey Bees			
Ferencik,N.	1961	0.072-0.624 ug/bee	Elemental As?,lethal dose
		0.12 ug/bee	Poisoning indicated
		0.0-0.091 ug/bee	Healthy bees
Debackere,M.	1972	1.0 ug/bee	Lethal dose
		0.5-0.37 ug/bee	Has been found to be lethal
		0.12 ug/bee	Proof of poisoning

[1]Atkins,E.L. Citation from George Grant Ballantine, d/b/a Cloverdale Apiaries, Plaintiff vs Anaconda Company, Defendant, In District Court of the Fifth Judicial District of the State of Montana, In and For the County of Jefferson, 1976.

[2]Unpublished data. Montana Department of Health and Environment, Chemistry Laboratory Bureau, Helena, Montana, 1974.

dusts from the early or ongoing operation of smelters (Knowlton, et al, 1950).

In the 1960's several reports of industrial poisoning of honey bees in Europe appeared. Svoboda (1960) stated that poisoning of honey bees occurred from factories that burned low-grade coal. She indicated that arsenic injury was severe within three to seven km of the damaging industry with pollen as the main source of poison (Anderson and Atkins, 1968). Lethal arsenic dose levels which have been reported in connection with industrial poisoning of honey bees are presented in Table 1. These values are in the range of 0.1 - 1.0 ug/bee of elemental arsenic.

There has been a history of beekills and similar difficulties within the Anaconda area of Montana. Swain and Harkins (1908) demonstrated that large quantities of arsenic were distributed from the Anaconda Smelter in Montana. The most favorable time for the accumulation of arsenic on plants was in the late summer and fall. In a previous study the main chimney of the smelter was found to discharge 59,270 lbs of arsenic trioxide a day (Harkins and Swain,1907).

Anaconda emission data for November 1979 states that 3.5% of a dust sample from the main stack was elemental arsenic. This figure can be converted to 0.241 tons of arsenic per day (personal communication from David Maughan, Montana State Department of Health and Environmental Sciences, Helena, Montana. April 1980). Approximately 1.8 lbs of As/hour from the main stack is cited by the February 28, 1979 particulate emissions data. In addition, data for this entire month indicates about 100 tons of unaccounted arsenic. The end

destination of this arsenic is unknown and has been assumed to be lost out of the converter building. Since the majority of ores for the Anaconda Smelter come from Butte, Montana it has been supposed that the emission of arsenic is fairly consistent throughout the year. The Environmental Protection Agency is ready now to list arsenic as a hazardous pollutant specifically in connection with copper smelters (personal communication from Mike Davenport, Environmental Protection Agency, Helena, Montana).

In 1975 a number of honey bee samples from the area were analyzed for arsenic. The following results were obtained:

Sample Date	Arsenic
6/5/75	0.28-0.40 ug/bee
7/17/75	0.16-0.35 ug/bee
9/2/75	0.27-0.45 ug/bee
10/2/75	0.37-0.68 ug/bee

The same sample sites were used for the four collection dates. These sites were Galle, Meyer, Warm Springs, Pond #3 and Spangler, Montana, all within 20 miles of the Anaconda Smelter. The arsenic concentrations reported above include the total range of concentrations found for all the samples collected on that date. (George Grant Ballantine, d/b/a Cloverdale Apiaries, Plaintiff vs Anaconda Company Defendant, In District Court of the Fifth Judicial District of the State of Montana, In and For the County of Jefferson, 1976).

CHAPTER III

MATERIALS AND METHODS

Oral Dose Tests

From June 12 to August 5, 1978 oral dose tests on the honey bee (Apis mellifera. L.) were conducted using arsenic trioxide (As_2O_3) and sodium arsenite ($NaAsO_2$). Five bee hives were moved from Manhattan, Montana to Fort Missoula in Missoula, Montana. Manhattan is a rural area with no known sources of arsenic pollution. Thus is was assumed that the honey bees were relatively arsenic free. At Fort Missoula, the hives were placed in a field owned by the University of Montana by the Clark Fork River. This area is located on the far south of town and is relatively isolated from cars, industry and other sources of contamination.

Foraging honey bees were collected by placing funnels leading into screened one gallon plastic bottles against the entrances of the hives (Figures 1 and 2). By blocking all other exits, bees leaving the hives were forced into the bottles. A bottle was assigned to each hive and used throughout the summer. Honey bees collected in this manner were mainly worker bees with the exception of a few drones. To minimize the loss of honey bees from overheating and shock, the bottles were shaded and periodically sprinkled with water throughout the collection period. The time required to collect the necessary number of bees varied depending on the weather and vigor of each hive. An effort was made to install the funnels during the early morning before the honey bees began to forage. Working in the

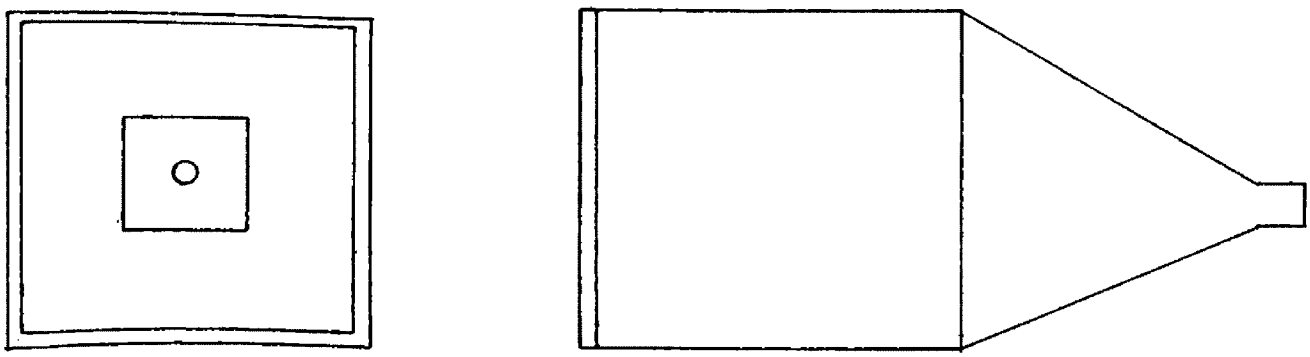


Fig. 1. Hive Collection Funnel. Front and side view.

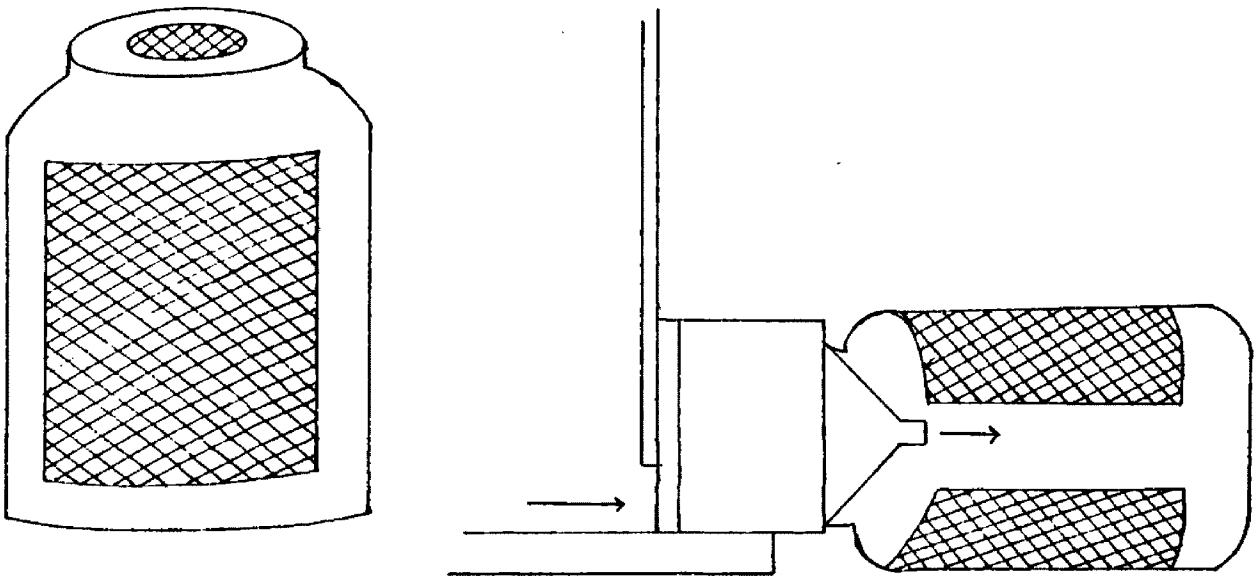


Fig. 2. Collection Bottle. Collection bottle and collection bottle attatched to hive.

morning also helped decrease the loss of honey bees from heat. As soon as a bottle appeared to be moderately crowded, it was removed from the hive entrance, covered with a screen top and placed into a metal ice chest. Here again the bottle was sprinkled with water to help keep the bees cool.

When collecting was completed, the ice chest of bottled bees was moved to the laboratory as soon as possible. This transfer period ranged from 30 to 45 minutes. At the laboratory, the bottles were quickly removed from the chest, placed in front of a fan and given more water. Then, as fast as possible, the honey bees were transferred to test cages and placed in the environmental chamber with feeding vials of sugar water.

A funnel was also used to transfer the bees to the test cages. This funnel had a large hole and was screwed onto the collection bottles. Bees were transferred by inverting the funnel into the cage and shaking the bottle (Figures 3, 4 and 5). The number of bees placed into each cage was estimated and varied from 13 to 598 bees. Numbers depended upon the quantity of bees collected from each hive. A small number of bees was left within each of the five screened collection bottles. These bottles were placed into a refrigerator to kill the honey bees. Later the dead bees were removed from the bottles, placed in labeled plastic bags and placed in a storage freezer. These samples represent nondosed bees from each colony and were labeled colony controls.

Conditions of the environmental chamber were as follows: temperature - 26.67^{o}C with a safety high of 43.3^{o}C and safety low

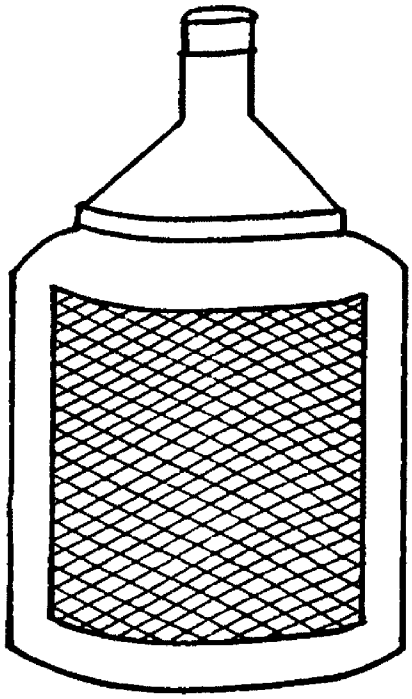


Fig. 3. Collection Bottle with Test Funnel.

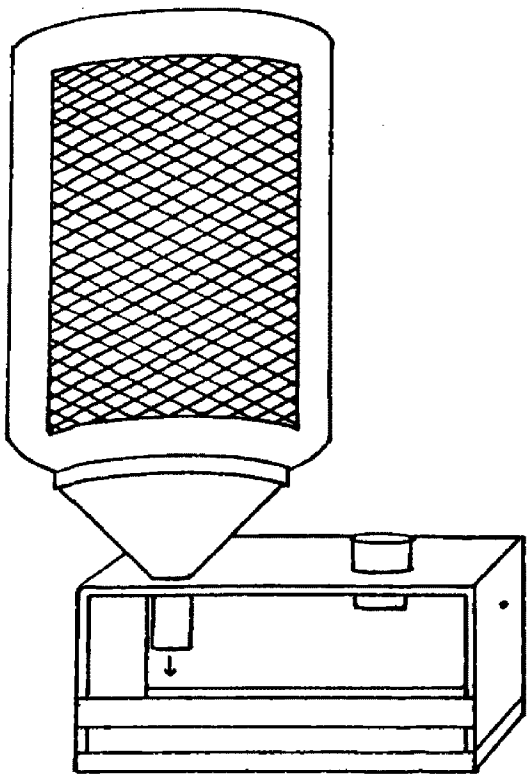


Fig. 4. Collection Bottle with Test Cage Funnel Attatched to Test Cage.

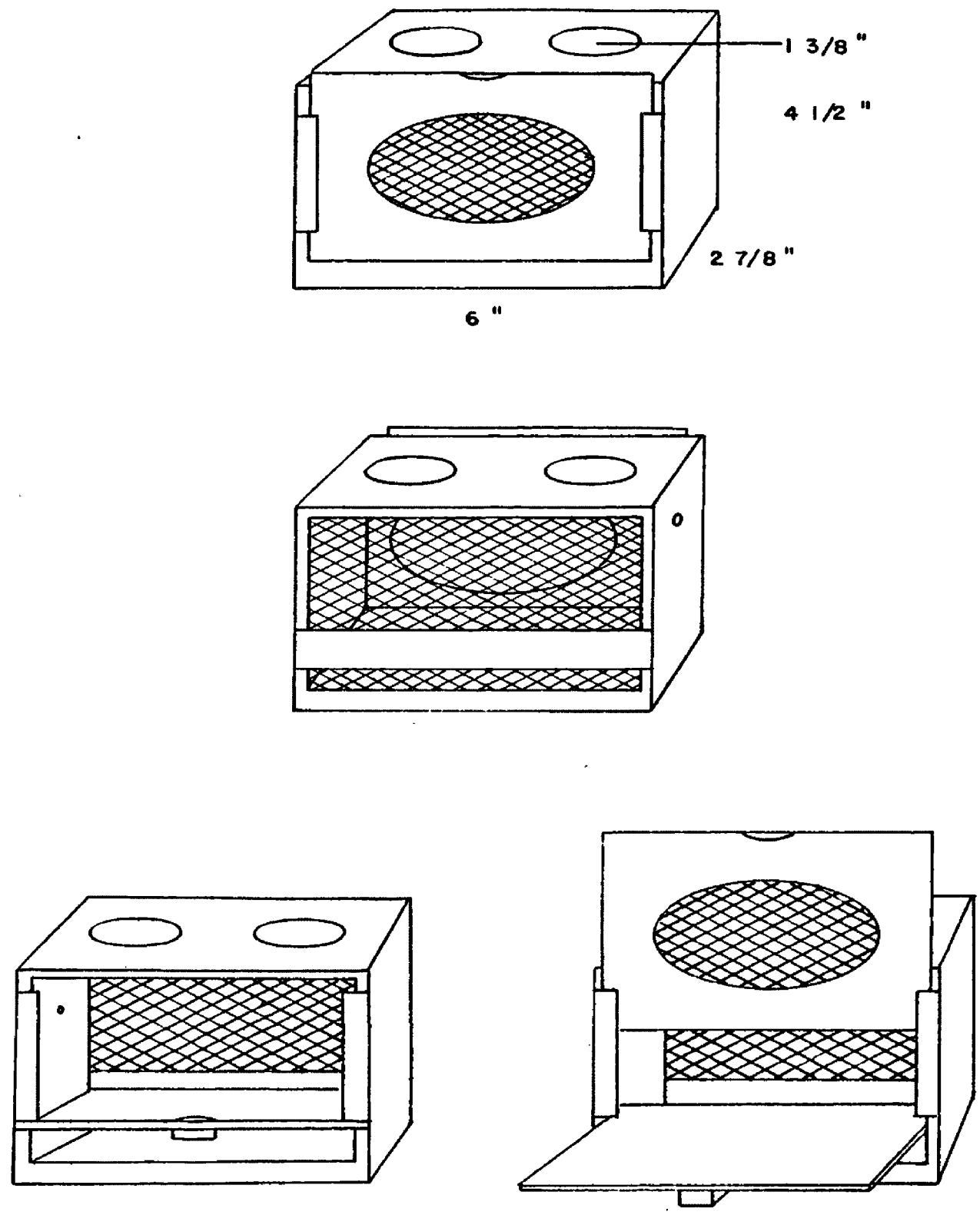


Fig. 5. Test Cage. Top: Front view. Middle: Back view.
Left Bottom: Inside showing false bottom.
Right Bottom: False bottom mechanism.

of 10°C; light period - 8:00 PM - 8:00 AM, dark period 8:00 AM - 8:00 PM. A study of the internal temperature of the environmental chamber indicates that the temperature fluctuates only by ± 2°C from the dial setting (personal communication from Steve Marvel, PhD candidate in botany, University of Montana, Missoula, Montana). A dish of water was placed at the bottom of the chamber to maintain humidity. There was no formal humidity control. Later due to problems with the environmental chamber shutting off too frequently, the safety temperature range was increased to a high of 45°C and low of 0°C. In addition, the dark and light periods were adjusted to facilitate the collection of dead bees. Honey bees are less easily aroused and active during or after a long dark period. Thus the dark period was scheduled to coincide as much as possible with the collection of dead bees. Light and dark periods were maintained in 12 hour intervals.

The cages were organized within the environmental chamber by colony and test number (Figure 6). Periodically the arrangement was changed to eliminate any variation due to placement within the environmental chamber. In addition, there was one control cage per colony. These bees were treated in the same manner as the poisoned bees. Usually three dose levels were run at the same time. Each dose level represents a test.

Eighteen oral dose tests were conducted. Table 2 lists these tests by number, chemical, date and theoretical dose. The theoretical dose was the predicted arsenic dose intended to be administered to the honey bees. These values assumed a 24 hour dose period and a 0.2

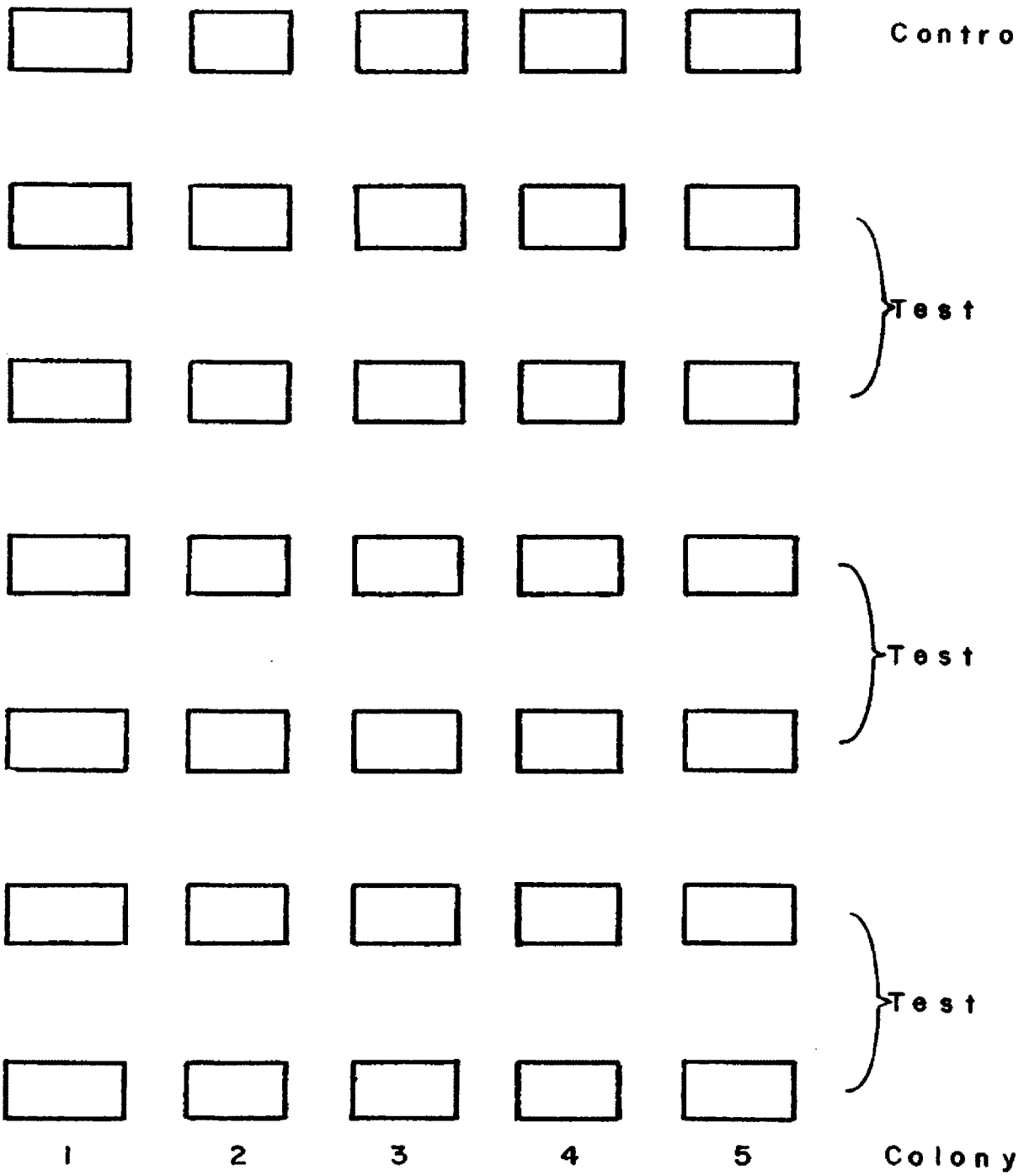


Fig. 6. Arrangement of Test Cages in Environmental Chamber.

TABLE 2

ORAL DOSE TESTS

Test Number	Chemical Compound	Date	Theoretical Dose
1	As_2O_3	June 12-17	0.5/3.0* ug/bee
2	"	June 12-17	0.07/0.42
3	"	June 12-17	0.01/0.06
4	"	June 25-July 1	3.00
5	"	June 25-July 1	0.42
6	"	June 25-July 1	0.06
7	$NaAsO_2$	July 3-8	0.50
8	"	July 3-8	0.07
9	"	July 3-8	0.01
10	"	July 10-15	10.00
11	"	July 10-15	5.00
12	"	July 10-15	3.00
13	"	July 17-22	8.00
14	"	July 17-22	7.00
15	"	July 17-22	6.00
16	"	July 30-August 5	4.00
17	"	July 30-August 5	2.00
18	"	July 30-August 5	1.00

* First value is for the 4 hour dose period, the second for the 24 hour dose period (4 hour dose period plus 20 hour dose period), Tests 1-3.

ml/bee/day consumption rate of arsenic sugar solution (Sonnet, et al, 1978). Furthermore the values are based on the fact that 49 ml of distilled water plus 98 ml of granulated sugar give 100 ml of sugar solution which was determined experimentally. The additional assumption was made that the honey bees consumed equal amounts of sugar solution. Whenever possible (about three fourths of the tests) at least two cages of bees were established per dose from each colony, ensuring duplication and adequate numbers for statistical analysis.

Caged honey bees were placed in the environmental chamber for 24 hours before dosing to allow for acclimation and any die off due to the transfer procedure. The sugar solution was removed for up to eight hours to ensure consumption of the arsenic sugar solution. Any bees which had died during the first 24 hours were removed and counted. Next, feeding vials containing the arsenic sugar solution were placed for 24 hours on the cages. Feeding vials consisted of one inch diameter plastic vials with pin holes in their bottoms.

The arsenic solutions were mixed in polyethylene bottles just before being administered. White granulated sugar, distilled water and technical grade As_2O_3 or $NaAsO_2$ were used. The general procedure involved making aliquots from high concentration stock solutions (30.6 ppm As_2O_3 and 3000 ppm $NaAsO_2$) which were then used to make the appropriate dilutions for the arsenic doses desired. Standard pipets were used to measure the arsenic solutions and distilled water, and a 100 ml graduated cylinder was used for the sugar. Feeding vials were weighed before and after the 24 hour dose period in order to determine the amount consumed. In addition, a control feeding vial of

sugar solution was placed in an empty cage in the environmental chamber during the dose period to record losses due to evaporation.

During the first three tests, the arsenic solution was administered for only four hours. When after two days, none of the expected die off had occurred, the arsenic sugar solution was readministered for an additional 20 hours. Thus for these tests in Table 2 there are two reported values, a four hour dose and a 24 hour dose. The assumption made was that honey bees which consumed the 20 hour dose contained arsenic from the four hour dose period. The majority of data has been based on the 24 hour dose period.

After 24 hours the arsenic sugar solution was replaced with regular sugar water. Dead honey bees were then collected and counted each day for up to six days after the 24 hour dose period. In addition the number of dying bees, defecation and general behavior of the honey bees was observed and recorded. Honey bees were considered dead if no response was received from prodding. Those which were too weak to fly or twitched when touched were defined as dying bees. Samples were differentiated by test number, colony number, cage number and date. All bee samples were placed in separate, labelled plastic bags and kept in a Sears Coldspot storage freezer at $-18^{o}C$ in the EVST Laboratory of the University of Montana.

On the last day of the test, any bees remaining alive were sacrificed by turning the environmental chamber up to $40^{o}C$ and removing the feeding vials. This method was not totally satisfactory since the honey bees became brittle at times and appeared to regurgitate some of their fluids. Freezing was tried but proved to be

inefficient due to the bees clustering behavior.

All feeding vials, cages and other equipment were washed between test sets. The vials or cages were first washed with alconox soap and water and rinsed well with hot tap water. Then all surfaces were rinsed with 1:1 concentrated HCl: distilled water, hot tap water and then with distilled water. The equipment was allowed to dry with interior portions protected on paper towels on the laboratory counter.

Tissue Residue Analysis

Frozen honey bee samples were analyzed for arsenic during the period June to November 1979. The analytical procedure required one gram samples, about 30 - 40 bees. In many of the dose tests, fewer than 30 bees died per day. Thus separate cage samples for each dose level had to be combined. Collections of dead bees made prior to administering arsenic contained sufficient numbers for analysis. Others were combined. When necessary samples of dosed bees were pooled by collection day. Only samples of the same test, colony, and cage number were combined. In some instances, large samples were subdivided for more then one analysis.

Twenty-six analysis sets consisting of 457 samples, were analyzed. Each set consisted of approximately 19-20 bee samples plus three blanks and four standards. When feasible, samples and controls from the same colony and test were analyzed as a set. In this manner sample arsenic concentrations could easily be adjusted for background arsenic of nondosed bees.

Two digestion methods were tried. The first was the classical wet oxidation method using nitric (HNO_3), perchloric ($HClO_4$) and

sulfuric (H_2SO_4) acids. The procedure and technique is one described and used by Dr. D.R. Neuman of the Agricultural Experiment Station at Bozeman, Montana (letter dated 1977 July 28 from Dennis R. Neuman, Animal and Range Sciences Department, Agricultural Experiment Station, Bozeman, Montana). This procedure is as follows:

1. Dry honey bees in a drying oven (45°C) for at least three days in watchglass covered beakers.
2. Weigh one gram of dried bees and place into a 125 ml Erlymeyer flask.
3. Add 30 ml of 3:2 mixture of concentrated HNO_3:$HClO_4$.
4. Let stand overnight.
5. Heat samples slowly to solubilize, then increase to reduce to one half volume.
6. Cool. Add 10 ml 1:1 mixture of concentrated HNO_3:H_2SO_4.
7. Heat to perchlorate fumes and continue to increase heat to about 500°F (210°C).
8. Continue to heat to dense white sulfate fumes.
9. Reduce to five ml.

This procedure is lengthy, taking 8-15 hours, but thorough.

The second method tried was a much faster procedure which has been utilized for digesting coniferous foliage (Behan and Kinraide, 1970). The procedure was slightly modified for honey bees as follows:

1. Dry honey bees in a drying oven (45°C) for at least three days in watchglass covered beakers.
2. Weigh one gram of dried bees and place into a 125 ml Erlymeyer flask.
3. Add 20 ml 3:2 mixture of concentrated HNO_3:$HClO_4$.
4. Let stand overnight.
5. Place flask on a hot plate preheated to about 500°F (210°C).

6. Heat for 20 minutes.

To compare the two procedures, an analysis was conducted using split samples from high dose tests. One set of samples was digested by Dr. Neuman's procedure, the second by the procedure described by Dr. Behan.

The digested samples were analyzed on the Instrumentation Laboratories Model 251 Atomic Absorption Spectrophotometer using a method of arsine generation described by Dr. Neuman (letter dated 1977 July 28 from Dennis R. Neuman, Animal and Range Sciences Department, Agricultural Experiment Station, Bozeman, Montana). This method is fairly sensitive and is able to detect as little as 0.3 ng arsenic (Siemer,et al,1976). The method and instrumentation will be described in greater detail later.

Table 3 lists the results of these tests. The assumption was made that a higher arsenic content indicated a more thorough digestion procedure. Thus the results favor method #1. Samples digested by this method have consistently higher amounts of arsenic then the corresponding samples digested by method #2. Furthermore, samples digested by Dr. Behan's method had more of an unknown, white, crystalline residue and appeared to have a thin film on the surface of the cooled digested sample. Therefore the honey bee samples were digested by Dr. Neuman's method.

The results of a comparison between oven dried and freeze dried samples also are presented in Table 3. Samples were freeze dried for 24 hours; splits were oven dried for three days. The freeze dried samples display a higher arsenic concentration, but were noticeably

TABLE 3

DIGESTION METHOD COMPARISON

Sample Number	Method #1*	Method #2*
184	1.18 ug/bee	1.08 ug/bee
188	0.95	0.97
194	0.96	0.79
291	0.85	0.78
301	0.78	0.52
302	0.88	0.80
303	0.02	0.00
305	0.96	0.66
316	0.26	0.06
321	0.25	0.11
327	0.37	0.11
331	0.48	0.14

Sample Number	Oven Dried	Freeze Dried
190	1.05 ug/bee	1.08 ug/bee
		0.91
191	1.13	1.75
		1.13

* Method #1 - Method described by Dr. Neuman
Method #2 - Method described by Dr. Behan

heavier and wetter then oven dried samples. Oven drying was chosen for a number of reasons. First the arsenic values of oven dried samples appeared reasonable, although slightly lower then freeze dried ones. Second the oven dried samples yielded greater analytical precision. Finally the use of the oven drier was much more convenient, allowing sufficient space to dry a whole analysis set at once.

A number of blanks and standards were utilized throughout the study. The following blanks were included: an acid blank which included all the digestion acids and analysis chemicals, an HCl blank, and a one gram sample of Standard Reference Material 1577 Bovine Liver from the National Bureau of Standards. The bovine liver standard was freeze dried for 24 hours prior to use. Arsenic standards were made the day of the analysis utilizing Varian-Techtron Arsenic Standard 1000 ppm Na_2HAsO_4 in a water matrix. They were calculated to bracket the estimated concentrations of the honey bee samples. These standard concentrations were 24 or 50, 100, 500, 800 or 900 ng/ml of arsenic.

All acids and reagents used were of reagent grade material. Blanks utilized during the analysis procedure helped to indicate possible arsenic contamination from these sources.

All glassware was washed in hot soapy water with alconox, rinsed ten times with hot tap water, rinsed with 50% concentrated HCl and rinsed again four times with distilled water. In addition, the digestion flasks were soaked overnight with sodium hydroxide in distilled water prior to washing. During the last half of the chemical analysis period and for every other analysis, these digestion flasks

were filled with sodium dichromate acid and left to stand overnight, prior to rinsing and filling with the sodium hydroxide solution. This washing procedure was necessary to eliminate a fine white residue which collected on the flasks after two to three digestion runs. It was assumed that the concentrated digestion acids prevented the loss of arsenic in this residue and that the film was from some component of the bee bodies.

After the samples were digested and cooled, they were filtered into 50 ml volumetric flasks that contained 15 ml concentrated HCl and one ml of 1% (w/v) KI. They were then left to cool and stand for one hour to allow the reduction of As^{+5} to As^{+3}. During this hour, the standards were prepared in 100 ml volumetric flasks with 30 ml HCl and two ml of KI. These were also allowed to stand at least one hour.

Three to six 20 ml aliquots of the standards and two 20 ml aliquots of the samples and blanks were then placed in plastic polyethylene reaction flasks. These flasks were covered with parafilm or plastic lids and transferred to the Chemistry Department of the University of Montana. Here they were analyzed on the Instrumentation Laboratories Model 251 Atomic Absorption Spectrophotometer.

A special stopper apparatus, illustrated in Figure 7, was attached to the AA. This apparatus allows the generation of arsine gas and its movement into the burner head. While the AA warmed up, nitrogen and hydrogen gas tanks were connected to the instrument,the pressure adjusted, the burner head positioning checked and 5% (w/v) $NaBH_4$ prepared.

The instrumental parameters were as follows:

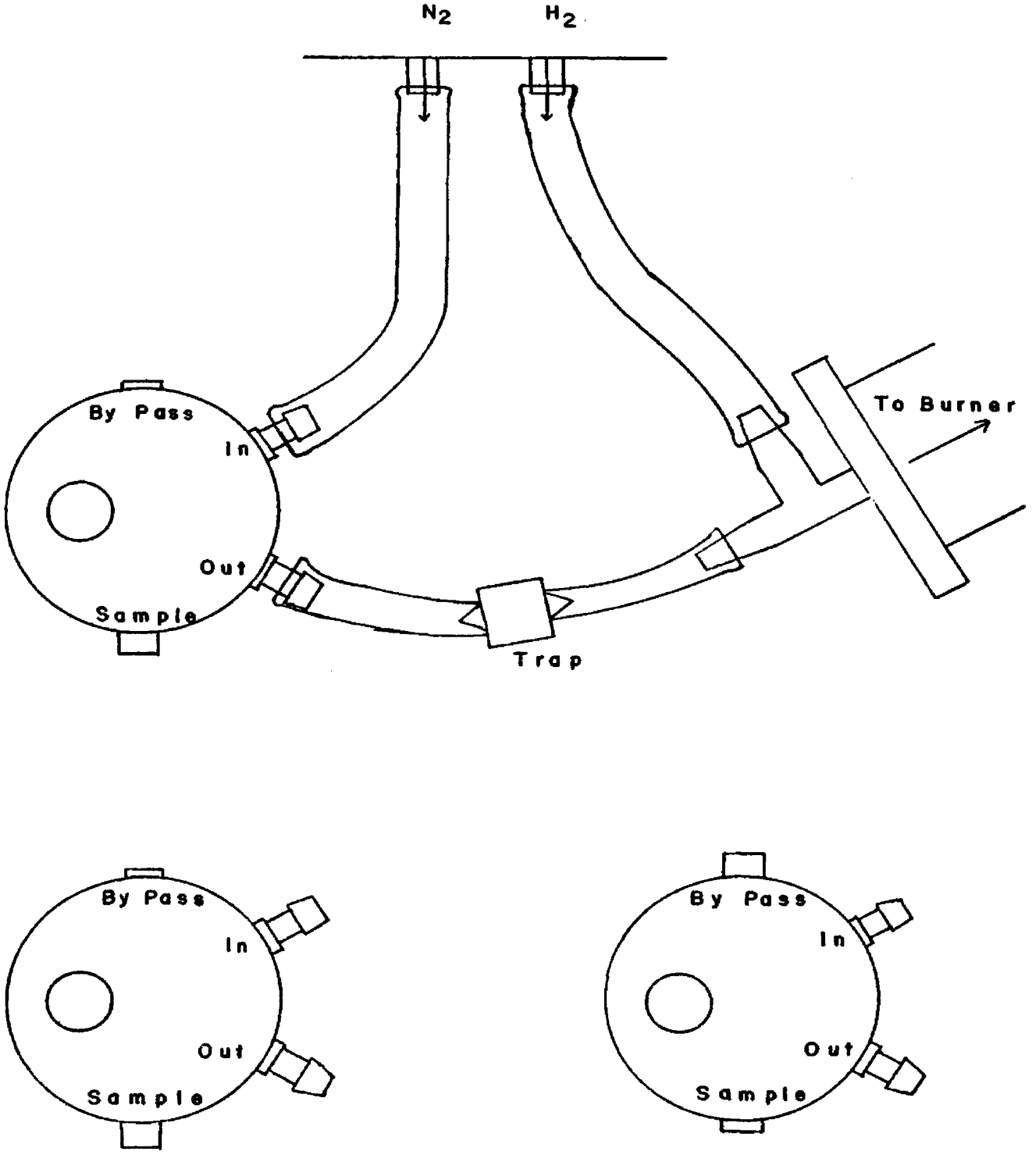


Fig. 7. Arsenic Stopper Apparatus. Left Top: Reaction Flask Stopper. Right Top: Aspirator Block. Left Bottom: Valve set to Sample. Right Bottom: Valve set to By Pass.

λ:	193.7 nm	mode:	automode 1/16 sec.
SLW:	1.0 nm	N_2:	23 cu ft/hr
HC:	10.0 mA	H_2:	6 cu ft/hr
HV:	800.0		

It was necessary to allow the instrument to warm up for at least 30 minutes to achieve sensitivity and stability. The chart recorder was set at 100 MV with a speed of one in/min.

A magnetic stir bar was placed in each reaction flask and the flask attached to the stopper system with the valve set at By Pass. The valve was then switched to Sample and five ml of 5% (w/v) $NaBH_4$ added thru the septum. $NaBH_4$ reacts violently with the sample, generating arsine gas which is swept out of the flask and into the flame by the nitrogen gas. A sharp absorbance peak is recorded in the presence of arsenic.

Standards were analyzed at the beginning, the middle and at the end of each analysis set. This was done to determine any drift and change in the sensitivity of the instrument. Blanks were analyzed at the beginning of the set. The analysis procedure on the AA usually took from two to three hours per set (about 59 sample aliquots).

Arsenic concentrations of the samples were derived from the recorder chart in the following manner. First a baseline was drawn through the analysis noise in such a way as to average it. Using peak height as the indication of arsenic concentration, the absorbance value for each sample was measured and recorded. Next the absorbance values of the standards were used to construct a concentration vs absorbance curve. Sample absorbance values were located on this curve and the corresponding concentration for this point noted. About 14% of the sample absorbances were greater then the highest standard. In

these cases a very approximate curve, based on proportionality, was drawn and used to estimate the arsenic concentrations of these higher samples. The resulting concentration values were then transformed to ug/bee of arsenic.

Procedural Tests and Results

In addition to the honey bee analysis there were several other related investigations which were conducted. One procedural investigation dealt with an unknown residue which appeared in the digested bee samples. This residue appeared in samples with and without arsenic. Observation of the residue under a dissection microscope showed clear long rectangular crystals. The crystals were geometrical and fairly uniform.

At first it was believed that some error was being made during the digestion procedure. Later, the same residue was obtained when similar honey bee samples were digested in Bozeman, Montana by Dr. Neuman. A quantity of the residue was obtained by digesting seven nondosed bee samples and filtering them with a Gucchi filter. Several tests were then made to determine the characteristics of the residue. The results of these tests are summarized in Table 4.

The first test involved the use of potassium dichromate as a color indicator of organic material. Five mg of potassium dichromate was placed into a sample which was then digested by the previously described method. Green indicates organic material, orange the completion of oxidation and total digestion. In addition to the above test, a small amount of a digested sample with residue was heated to dryness. Organic material in the presence of perchloric

TABLE 4

UNKNOWN RESIDUE

Test	Result	Conclusion
Potassium Dichromate	Orange	Nonorganic
Digestion to Dryness	No charring	Nonorganic
$Na_3Co(NO_2)_6$	No reaction or precipitate	Not potassium
Solubility		
0.1034 g unknown	Did not dissolve with 1000 ml distilled water	Not very soluble in distilled water
0.0167 g unknown	Majority of unknown dissolved, can just barely see particles with 250 ml distilled water	

TABLE 5

VERIFICATION TEST

Sample*	Concentration*
0.000 ug/ml	0.02 ug/ml
0.005	0.02
0.010	0.00
0.015	0.01
0.025	0.02
0.050	0.05
0.100	0.17
0.300	0.42
0.400	0.57
0.500	0.68
0.900	0.78

r = 0.9565 Standard Error = 0.0790
y = 0.0371 + 1.0052x

* Sample - 1 g of ground,control honey bees with the designated arsenic concentration added.
Concentration - all values adjusted by the 0.000 sample to account for background arsenic.

acid produces a char in this situation. The potassium dichromate turned orange in the digested samples and there was no charring, indicating that the unknown is a nonorganic substance.

The presence of potassium was also tested for. A small amount of the unknown was dissolved in a small test tube. An equal amount of KCl was treated in the same manner. Two drops of $Na_3Co(NO_2)_6$ were then added to each sample. The presence of potassium is indicated by a yellow-brown precipitate. Such a precipitate did not occur in the test tube with the unknown. Therefore this unknown does not contain potassium.

Dilution tests were conducted. In the first test 0.1034 g of the residue was placed in a 1000 ml erlymeyer flask and distilled water added until it dissolved. This test was unsuccessful with 1000 ml of water being added without any visible dissolution. A second test was run using only 0.0167 g of the residue. The unknown residue proved to be fairly insoluble in distilled water. Even the 0.0167 g sample when diluted with 250 ml of water still had particles of the unknown visible.

Another procedural investigation involved the analysis of honey bee samples with known amounts of arsenic. This test was meant as a verification and validation of the chemical analysis procedure. Nondosed and control honey bees were placed in clean beakers and dried for three days in a drying oven. These bees were ground together and mixed, creating as homogenous a sample as possible. One gram samples were weighed, placed in 125 ml erlymeyer flasks and various amounts of arsenic solution added. These samples were then digested and analyzed. The arsenic concentrations used were 0, 5, 10, 15, 25, 50, 100, 300,

400, 500, and 900 ng/ml. All the usual blanks and standards were included.

The verification test demonstrates that the chemical analysis procedures and techniques are very reliable. Table 5 (p. 36) reports the results and the statistical analysis of these results. The correlation coefficient r, 0.9565, is very high and positive; and the standard error, 0.0790, low; this indicates that the arsenic concentration derived from the chemical analysis corresponds closely to the concentration of arsenic placed in the sample. Thus it is clear that little arsenic is lost throughout the digestion and atomic absorption spectrophotometer analysis. Furthermore, the results certify that the interpretation of the recorder charts did not have a noticeable effect on the arsenic concentration values.

An investigation was conducted to study the change in the concentration of arsenic, if any, in the sugar solutions before and after 24 hours in the environmental chamber. Attempts were made to duplicate the procedure of the oral dose tests as closely as possible. New stock solutions of 30.6 ppm As_2O_3 and 3000 ppm $NaAsO_2$ were made. Next the following arsenic sugar solutions were prepared:0.01, 0.07, 0.5 ug/bee As_2O_3 and 0.07, 0.5, 3.0, 5.0 and 10.0 ug/bee $NaAsO_2$. The same chemical calculations used for the oral dose tests were used to prepare these solutions. The calculations were based upon the assumption of 0.2 ml/bee/day and a 100 ml volume of sugar solution from 98 ml granulated sugar and 49 ml distilled water. A one ml aliquot of 10.0 ug/bee $NaAsO_2$, two ml aliquots of 0.5 ug/bee As_2O_3 and 3.0, 5.0 ug/bee $NaAsO_2$; 10 ml aliquots of 0.01, 0.07 ug/bee As_2O_3

and 0.5, 0.07 ug/bee $NaAsO_2$ and 20 ml aliquot of 0.01 ug/bee $NaAsO_2$ were placed in 125 ml erlymeyer flasks and digestion acids added. These samples were left to stand in a protected fume hood until the remainder of the test samples were ready to be digested. It was assumed that the concentrated digestion acids would prevent any loss of arsenic into the glassware. The remaining portions of each of the sugar solutions were used to fill feeding vials which were placed in empty bee cages in the environmental chamber. These samples were left for 24 hours. The environmental chamber was set at the same temperature and light regime used during the oral dose tests. After 24 hours, the feeding vials were removed and similar aliquots, as described above, prepared for analysis. In addition, upon emptying, several of the feeding vials were rinsed quickly with distilled water and then rinsed with five to ten ml of concentrated HCl which was placed into erlymeyer flasks to be treated as separate samples. All samples were then carried through the standard chemical analysis procedure.

Table 6 contains the results of this investigation. The expected concentration of arsenic is derived from the theoretical dose values and an assumption of the 0.2 ml/bee/day. A high correlation coefficient value of 0.9994 is given for the relationship between the expected concentration and the concentration from chemical analysis of the before 24 hour samples. Thus 99.9% (r^2 x 100) of the chemical analysis concentration is explained by the expected concentration. From this high correspondence, it is evident that the density, viscosity and other characteristics of the sugar solution have no major effect on

TABLE 6

ARSENIC SUGAR SOLUTION TEST

Sample Theoretical Dose	Expected* Concentration	Concentration from the Chemical Analysis	
		Before 24 hrs	After 24 hrs
As_2O_3			
0.06 ug/bee	0.30 ppm	0.34 ppm	0.48 ppm
0.42	2.10	3.03	3.60
3.00	15.00	13.20	15.28
$NaAsO_2$			
0.07	0.35	0.70	0.73
0.50	2.50	2.02	2.24
3.00	15.00	14.38	15.50
5.00	25.00	22.50	22.50
10.00	50.00	45.00+	45.00+
As_2O_3			
0.42 vial rinse	low	-	0.04
3.00 vial rinse	low	-	0.02

[1] Calculated concentration of arsenic based upon the following assumption: 0.2 ml/bee/day. Therefore 0.06 ug/bee 0.06 ug/0.2 ml 0.30 ug/ml.

the arsenic concentration within the solution.

There is a strong positive correlation, r = 0.9970, between the arsenic concentration of samples placed for 24 hours in the environmental chamber and those which were not. The concentration before 24 hours explains 99.4% of the concentration of samples after 24 hours. There is a 13-28% increase of arsenic in the environmental chamber samples of arsenic trioxide and a 3-9% increase in the sodium arsenite samples. Evaporation ranged from 0.338 ml to 0.662 ml from a 35-40 ml total volume which could easily explain most of this increase in arsenic concentration. Therefore there was no significant loss of arsenic from the arsenic sugar solution due to the time in the environmental chamber. We are assured that the honey bees were exposed to a constant concentration of arsenic throughout the 24 hour dose period.

The concentration values of the rinses of the vials for the 0.42 and 3.00 ug/bee As_2O_3 solutions are 9 .8% and 0.5% of the total concentrations. The first percentage appears fairly high and could be due to the incomplete preliminary rinse of the feeding vial or to actual absorbance of arsenic on the vial wall. An incomplete preliminary rinse would leave a trace amount of As sugar solution within the vial which would then appear in the chemical analysis of the rinse. Since there was a high correlation between the expected concentration and the chemical analysis concentration, and a low rinse concentration; absorbance of arsenic onto the walls of plastic vials and glassware does not appear to be an important factor, except perhaps for the very lowest concentrations.

The final investigation was an analysis of the six arsenic trioxide and sodium arsenite stock solutions which were still on hand. These stock solutions were as follows:

As_2O_3:	30.6 ppm 3/8/79
	30.6 ppm 11/15/79
$NaAsO_2$:	1000 ppm
	1000 ppm 6/7/79 acidified
	3000 ppm 7/3/78
	3000 ppm 11/15/79

500 ng/ml (0.500 ppm) solutions were prepared from each stock solution and analyzed on the atomic absorption spectrophotometer using the arsine generation apparatus. In addition 30 ml of concentrated HCl rinsed from each empty and lightly rinsed stock solution flask was analyzed.

The analysis of the stock solutions suggests that they are fairly stable. As illustrated in Table 7, the expected concentrations of the aliquots analyzed and the chemical analysis results are reasonably close. The 3000 ppm 7/3/78 sodium arsenite solution is the only exception with a very high value of arsenic of 0.828 ppm. This exception could be a normal random error or due to inaccurate preparation. Notice that the more recently made stock solutions have actual arsenic concentrations slightly closer to the expected concentrations. Thus some aging is indicated but not enough to cause marked error in the results of the oral dose tests. Furthermore, the tests were conducted during the three months after the solutions were made and as seen these solutions are still fairly stable after about a year to a year and a half after they were made.

Arsenic trioxide is generally an insoluble compound. It took three to six hours of constant stirring over a hot plate to dissolve

TABLE 7

STOCK SOLUTION TEST

Sample	Expected Concentration	Actual Concentration	% of Solution
As_2O_3			
30.6 ppm 3/7/79	0.50 ppm	0.37 ppm	
30.6 ppm 11/15/79	0.50	0.55	
30.6 ppm 3/7/79 flask rinse	-	0.02	0.06
30.6 ppm 11/15/79 flask rinse	-	0.02	0.08
$NaAsO_2$			
1000 ppm	0.50	0.58	
1000 ppm 6/7/79	0.50	0.50	
3000 ppm 7/3/78	0.50	0.83	
3000 ppm 11/15/79	0.50	0.52	
1000 ppm flask rinse	-	0.87	0.09
1000 ppm 6/7/79 flask rinse	-	0.89	0.09
3000 ppm 7/3/78 flask rinse	-	0.90+	0.03
3000 ppm 11/15/79 flask rinse	-	0.90+	0.03

even the small amount of arsenic trioxide needed to prepare one liter of 30.6 ppm As stock solution. There is a 10-26% difference between the expected and actual concentrations of the arsenic trioxide stock solutions. Considering the number of steps and calculations in the analysis procedure this difference is not that unreasonable. Thus we can be fairly assured that the arsenic trioxide compound was totally dissolved during preparation.

HCl rinses of the stock solution flasks were also analyzed. These appear fairly high, ranging from 0.02 to 0.90+ ppm, but in comparison to the total solution concentration, they represent only a small percent, 0.089% or less, of the arsenic present. It is assumed that these rinses identify the arsenic that has been absorbed on the glass of the storage flasks. Therefore the absorption of arsenic on the stock solution flasks is negligiable.

Statistical Analysis

A number of calculations and statistics were derived from the data. One of the first calculations made was the actual concentration of arsenic administered to the honey bees (calcconc). This value was computed from the density of the arsenic sugar solutions, the weight of arsenic sugar solution consumed and the number of honey bees dosed. The density was obtained by weighing three to six 10 ml volumes of each solution. By dividing mass by volume, density values were calculated and then averaged to attain an overall value. These density values were used to determine the volume of arsenic sugar solution consumed by dividing the weight of the arsenic sugar solution consumed by density. The volume was divided by the number of dosed bees

and multiplied by the concentration of the solution administered to obtain the arsenic dose in ug/bee.

Mortality response graphs were constructed using the raw oral dose test mortality data. Two sets of graphs were produced. The first set plotted collection day vs % dosed dead bees/day and the second set collection day vs the accumulative value of % dosed dead bees/day for each individual cage of each dose test. Thus each test has a separate graph of each type, depicting the mortality response of each cage of the honey bees.

Median lethal dose values (LD_{50}) were calculated by the Miller and Tainter method as described by H.C. Batson in An Introduction to Statistics in the Medical Sciences. LD_{50} values were obtained for the pooled data and for each individual colony. To standardize the data, four days after the 24 hour dose period were used as the test period for these calculations. The procedure is as follows:

1. For each test calculate the total volume of fluid consumed and multiply it by the concentration of arsenic used.
2. Total the number of dosed bees for each test (T).
3. Calculate mean dose/bee by dividing the result of step one by step two.
4. Total the number of dead bees collected for each test (D).
5. Calculate D/T.
6. Calculate %D = D/T x 100.
7. Assign probit values. Table provided by H.C. Batson.
8. Plot the probit values as the linear ordinate values on semilog graph paper with mean dose/bee on the logarithmic abscissa.
9. Using inspection and a straight-edge draw the best fitting line.

10. Draw a vertical line through the point where the dosage-response curve crosses the probit five line and note the point where the abscissa is intersected. This point is the estimated LD_{50}.

The standard error was calculated from the following formula: $SE_{LD50} = \frac{2s}{\sqrt{2N}}$ where 2s equals the difference between the values of x corresponding to probit four and six, and N is the total number of test organisms in the groups included in the range of 3.5 and 6.5 probits. 95% confidence intervals were also calculated from the following equation: 95% CI = $X \pm 1.96$ (SE). The colony median lethal doses were derived in the same manner, with the data divided by test and by colony.

SPSS, the Statistical Package for the Social Sciences, was used to conduct a number of statistical analyses of the data. Alpha less then or equal to 0.05 was used as the significance level for all statistical inferences. An investigation of the relationship between "calcconc", the arsenic concentration calculated from the volume of arsenic consumed and considered the actual dose administered to the honey bees, and "conc", the arsenic concentration calculated from the tissue analysis was made using the Scattergram program. This program creates a scattergram of the data and applies a simple oneway correlation test. The correlation values r, r^2, standard error and significance levels are reported. The variables calcconc and conc were used to represent the laboratory or oral dose test results and the tissue residue results, respectively.

In addition, the influence of the colony and test number on calcconc, conc, proportion of dead bees per day and arsenic liquid consumption was investigated. The SPSS programs utilized during this statistical analysis were ANOVA, Oneway, and Scattergram.

Analysis of variance analyses were run to study the effect of collection day on the tissue residue value (conc) and the effect of the number of honey bees in the cage by colony and by test on the proportion of dead bees per day. Furthermore, general statistics were summarized for each test for the initial number of bees per cage, the number of dosed bees per cage, and the number of dead bees collected each day. These statistics were studied to see if any general trends are indicated.

CHAPTER IV

RESULTS AND DISCUSSION

Mortality Response Graphs

Appendix A and B contain the % dosed dead bees/day and accumulative % dosed dead bees/day mortality response graphs. Each line of each graph illustrates the response of a particular cage and group of honey bees. Both the % dosed dead bees/day and accumulative graphs portray the general response to the arsenic poisoning. Definite graph configurations are associated with the different dose levels. Variation is also indicated between bee cages and colonies. Possible factors are the number of bees per cage, the age of the honey bees, the vigor of the colonies, adequacy of food and the overall tolerance of the honey bees to arsenic and conditions of the test. There are no noticeable or consistent trends between colonies or individual bee cages.

The % dosed dead bees/day graph for Test 10 (p. 111), a high dose test, starts high, comes down with a sharp negative slope, then evens off at a steady low level. Test 13 (p. 115) also follows this general configuration but not as dramatically. Medium doses (Tests 1, 4, 11 thru 12, 14 thru 18) have graphs which jump up and down with a steady die off throughout the test period. In both the control and low tests (Tests 2 thru 3, 5 thru 9) the graph remains low and only has a sharp positive die off at the end of the test when the bees are sacrificed. In general the low dose tests have a slightly higher % dosed dead bees/day then the controls.

The graphs of accumulative % dosed dead bees/day appear more

detailed and informative. Here too there are definite configurations corresponding to dose. High doses, 0.65 - 0.49 ug/bee, create an open convex curve located at the top of the graph. A greatly flattened out convex curve is also seen in the graphs of the medium high dose levels, 0.47 - 0.46 ug/bee. These curves have a gradual slope. Medium low doses, 0.46 - 0.12 ug/bee, form a concave line. As before, the low doses, 0.09 - 0.001 ug/bee, and controls remain fairly level and then increase at the end of the tests. The controls are slightly different, showing an initial die off, then little response until the bees are sacrificed.

Arsenic trioxide was used for the first six oral dose tests. The % dosed dead bees/day graphs (Appendix A) indicate that there may be a consistently higher response from colony four during Tests 4-6 which represent calculated actual doses of 2.28, 0.33, and 0.04 ug/bee. This trend is slight and is not seen in other tests. The trend is also found in the number of dying bees data (Table 9, p. 53-54).

As described, a 4 hour and 20 hour dose period was administered during the first three tests. The accumulative graphs (p. 124-126) show a definite increase in the die off rate after the 20 hour dose. It is also shown that the mortality rates of these three tests do not match those of the following three tests which duplicate the same theoretical levels with 24 hour dose periods (p. 128-130). The tests with 2 dose periods appear to have a slightly greater response. This greater response becomes more noticeable at the lower dose levels. The number of dying bees also illustrates this relationship. Furthermore, the graphs show that the lower dose levels, 0.33 and 0.04

ug/bee, are practically the same as the control.

Sodium arsenite was used for the remaining oral dose tests. Here both the % dosed dead bees/day (Appendix A) and accumulative % dosed dead bees/day (Appendix B) indicate that Tests 7 - 9 (p. 132-134) are very similar to the controls. Both have flat graphs with very little mortality shown. Tests 7 - 9 represent calculated actual doses of 0.04, 0.01 and 0.001 ug/bee. The accumulative % dosed dead bees/day graphs show a number of additional facts. First, more variation between bee cages or groups of honey bees is indicated. This variation appears even greater in the tests above the calculated actual dose of 0.05 ug/bee. Second, it appears that the bees of Tests 16 through 18 (p. 144-146) are more sensitive to the arsenic poisoning. These tests showed slightly higher mortality response than tests of similar but higher dose levels. For example,Test 16 (p. 144) with a calculated dose of 0.34 ug/bee has a higher response then Test 11 (p. 137) with a dose of 0.46 ug/bee. Possible conditions which may contribute to this response are the age of the honey bees, colony viability and the number of bees per cage.

Tests 16, 17 and 18 were the last tests conducted, which was during the end of July. Fewer bees were used per cage than in previous tests. There was some evidence that the honey bees were more sensitive to handling at this time. With these three tests it was more difficult to minimize bee loss during the transfer process from the field. Heat prostration is a possible factor, although these bees were given the same cooling treatment as the honey bees of other tests.

Table 8 summarizes the sum values for the number of dead bees

TABLE 8

GENERAL STATISTICS - TOTAL NUMBER OF DEAD BEES COLLECTED BY COLLECTION DAY AND TEST NUMBER

Test	Actual* Dose	Day1	Day2	Day3	Day4	Day5	Day6	Day7
As_2O_3								
1	0.22	204	9	34	51	93	22	20
2	0.09	162	4	5	3	5	17	141
3	0.09	231	2	3	9	8	12	151
TC1-3	0.00	102	56	1	3	5	4	102
4	2.28	1000	25	149	265	325	179	343
5	0.33	1513	10	4	4	28	42	926
6	0.04	845	6	2	5	10	53	924
TC4-6	0.00	641	11	0	1	5	8	262
$NaAsO_2$								
7	0.05	476	14	10	-	34	729	-
8	0.01	479	10	19	-	43	671	-
9	0.001	308	9	13	-	29	600	-
TC7-9	0.00	245	8	3	-	11	369	-
10	0.65	367	1130	405	162	52	47	-
11	0.46	332	343	406	220	-	1025	-
12	0.35	358	128	317	311	-	1863	-
TC10-12	0.00	112	29	17	17	-	743	-
13	0.49	363	579	534	228	147	55	-
14	0.47	333	264	258	215	256	372	-
15	0.46	509	313	452	301	281	274	-
TC13-15	0.00	151	21	23	62	100	489	-
16	0.34	952	88	120	92	70	36	40
17	0.19	891	23	14	29	61	38	207
18	0.12	983	19	4	6	36	22	401
TC16-18	0.00	467	7	5	7	9	25	297

* ug/bee
TC - Test Control

collected for each day during the oral dose tests. By comparing the calculated dose to the pattern of mortality as shown by the Day2 through 7 statistics, the general mortality responses as illustrated by the mortality response graphs are seen. For sodium arsenite, high doses of 0.65 to 0.49 ug/bee show sharp die off rates for Day2 and 3. This die off continues, declining rapidly until there are only a small number of honey bees left at the end of the test. Moderate doses of 0.47 to 0.19 ug/bee have a steady and consistent die off rate throughout all the collection days. Doses of 0.12 to 0.01 ug/bee demonstrate a low die off rate similar to, but slightly higher then the controls. The very low dose of 0.001 ug/bee is fairly indistinguishable from the control in terms of the actual numbers of dead bees. These trends are also evident during the arsenic trioxide tests with the exception of the highest dose, 2.28 ug/bee, which shows a steady, increasing die off rate, instead of a sharp die off and decline. This exception may be due to the greater number of honey bees used for this test.

Number of Dying Honey Bees

Table 9 summarizes the data for the number of dying honey bees in relation to the number of honey bees dosed recorded throughout the oral dose tests. Dying bees were very noticeable and appeared to go through a number of stages of incapacitation. First the honey bees would become less active and lose the power of flight. Later such bees would only move their legs and tongues. Finally, dying bees would be so weak that they would only respond when prodded. The sequence of symptoms corresponds well to the literature . Copious

TABLE 9

DYING HONEY BEES STATISTICS

Test	Mean Calcconc	Colony	Number of Dying Bees[1]	% Dying Bees[2]	# Total Dying Bees	% Total Dying Bees[3]
1	0.22 ug/bee	1	3	3.09		
		2	1	3.45		
		3	3	6.12		
		4	4	16.67	11	5.82
2	0.09	1	1	1.75		
		2	0			
		3	0			
		4	4	14.28	5	3.01
3	0.09	1	2	3.17		
		2	1	3.45		
		3	0			
		4	1	2.38	4	1.65
TC1-3	0.00	All	0			
4	2.28	1	6	1.17		
		2	10	2.16		
		3	5	6.85		
		4	20	8.55		
		5	1	7.14	42	3.24
5	0.33	All	0			
6	0.04	1-3	0			
		4	2	0.93	2	0.20
TC4-6	0.00	All	0			
7	0.05	1	1	0.26		
		2	1	0.70		
		3-4	0		2	0.25
8	0.01	1	1	0.37		
		2-5	0		1	0.13
9	0.001	1	1	0.34		
		2-5	0		1	0.15
TC7-9	0.00	All	0			
10	0.65	1	15	9.20		
		2	137	22.42		
		3	11	7.86		
		4	67	10.63		
		5	32	12.12	259	14.32
11	0.46	1	22	7.28		
		2	41	7.06		
		3	21	18.92		
		4	57	7.24		
		5	31	14.35	172	8.61

TABLE 9

Test	Mean Calcconc	Colony	Number of Dying Bees	% Dying Bees	# Total Dying Bees	% Total Dying Bees
12	0.35	1	15	3.54		
		2	14	2.39		
		3	2	1.94		
		4	74	6.31		
		5	17	5.04	122	4.65
TC10-12	0.00	1-3	0			
		4	1	0.30		
		5	0		1	0.13
13	0.49	1	47	10.95		
		2	52	17.27		
		3	23	32.39		
		4	114	26.21		
		5	34	10.97	270	17.46
14	0.47	1	63	13.64		
		2	15	3.93		
		4	44	13.41		
		5	21	10.77	143	10.46
15	0.46	1	59	11.77		
		2	26	7.26		
		4	76	15.51		
		5	18	6.52	179	11.01
TC13-15	0.00	1	10	6.94		
		2-3	0			
		4	1	0.43		
		5	2	2.10	13	1.87
16	0.34	1	4	16.00		
		2	9	14.28		
		4	19	12.50		
		5	29	16.02	61	14.49
17	0.19	1	2	5.40		
		2	1	2.32		
		4	5	4.27		
		5	4	2.28	12	3.22
18	0.12	1	0			
		2	4	3.88		
		4	0			
		5	1	0.40	5	1.02
TC16-18	0.00	1	3	4.22		
		2	1	2.00		
		4	0			
		5	1	0.70	5	1.43

[1]24 hour dose only

[2]% dying bees in relation to the number of bees dosed for the specified colony and test.

[3]% of total number of dying bees in relation to the total number of bees dosed for the specified test.

defecation has been reported in some instances of heavy arsenic poisoning in the field. Although occasional yellowish drops of fluid were seen on the cage screens, there was no noticeable defecation by the dosed honey bees.

There is a definite increase of the number of dying bees with an increase in dose. Test 10 with a calculated dose of 0.65 ug/bee had a total of 14.32% number of dying bees while Test 17 with a dose of 0.19 ug/bee only had a 3.22% total number of dying bees. Some variation is indicated among the higher doses, where one dose may have a lower number of dying honey bees then a slightly lower dose. For instance, Test 16 with a dose of 0.34 ug/bee had 14.49% total number of dying bees while Test 12 with a 0.35 ug/bee dose has only 4.65% total number of dying bees. Another result is that the majority of low doses can be distinguished from the controls by the slightly higher % of dying bees. While the control had no dying bees, Tests 7 - 9 with very low calculated doses had at least one or two. Thus even though the mortality of the honey bees during these tests does not distinguish them from controls, there is some arsenic effect suggested.

The Actual Dose Administered

Using the volume of arsenic sugar solution consumed, the arsenic concentration administered and the number of dosed honey bees, the actual dose received by the honey bees for each cage was calculated. This calculated dose is labeled calcconc, an abbreviation for calculated concentration of arsenic. Table 10 presents the main calculated concentration, standard error, and standard deviation of

TABLE 10

THE CALCULATED CONCENTRATION OF THE ARSENIC DOSE

Test	Mean Calcconc	Standard Error	Standard Deviation	Theoretical Dose*
As_2O_3				
1	0.22 ug/bee	0.15	0.40	3.00 ug/bee
2	0.09	0.04	0.12	0.42
3	0.09	0.07	0.21	0.06
4	2.28	0.30	1.62	3.00
5	0.33	0.08	0.35	0.42
6	0.04	0.01	0.04	0.06
$NaAsO_2$				
7	0.05	0.01	0.05	0.50
8	0.01	0.00	0.01	0.07
9	0.001	0.00	0.00	0.01
10	0.65	0.06	0.35	10.00
11	0.46	0.05	0.26	5.00
12	0.35	0.04	0.23	3.00
13	0.49	0.05	0.30	8.00
14	0.47	0.04	0.25	7.00
15	0.46	0.04	0.25	6.00
16	0.34	0.07	0.30	4.00
17	0.19	0.05	0.19	2.00
18	0.12	0.03	0.12	1.00

* 24 hour dose only.

the mean for each dose test. One of the first noticeable facts is the definite difference between the theoretical dose and the calculated dose. For example, the theoretical dose for Test 10 is 10.00 ug/bee while the mean calculated dose for this test is only 0.65 ug/bee. As one may recall, the theoretical dose is the amount of arsenic contained in the arsenic sugar solution computed from an extimated 0.2 ml/bee/day and 100 ml volume of sugar solution made from 49 ml distilled water and 98 ml granulated sugar.

There are a number of possible causes for the difference. First the consumption of the arsenic sugar solution varied and did not coincide with the assumption of 0.2 ml/bee/day. In actuality, the average amount of arsenic sugar solution consumed per bee was 0.0379 ml of As_2O_3 and 0.0292 ml of $NaAsO_2$ during the 24 hour dose period. The number of honey bees per cage may decrease feeding. Often the cages were moderately crowded (200 - 300 bees) and, in a few instances very crowded (500 - 600 bees). Such crowding could reduce sugar solution consumption due to inactivity, interbee feeding and increased stress. Another possible influence is the arsenic dose level. More toxic and higher doses of arsenic could incapacitate honey bees so quickly that they are unable to consume much arsenic sugar solution. This hypothesis is supported by the actual volumes consumed. As reported, honey bees ate less sodium arsenite, the more toxic solution.

The calculated concentration values for arsenic trioxide seem to be variable. The fact that the arsenic trioxide tests were the first to be run may contribute to this variability. Similar

variability is found throughout all the arsenic trioxide results. Generally the calcconc values correspond in magnitude to the theoretical doses. For example, Test 7 has a theoretical dose of 0.50 ug/bee and a calculated dose of 0.05 ug/bee while the theoretical dose for Test 13 is 8.00 ug/bee with a calculated dose of 0.49 ug/bee.

Sodium arsenite appears to be much more reliable and consistent. Here there is a definite correspondence in magnitude with low to high doses. Notice also that the calcconc values (0.47 - 0.34 ug/bee) for middle ranges (7 - 3 ug/bee) of the theoretical doses tend to be very close together. Thus it would appear that closely related theoretical dose levels, for example 5.00 and 6.00 ug/bee, will administer very similar or the same dose to the honey bees.

For all tests the standard deviation of the calcconc mean is fairly large. This indicates that the range of calcconc values within each test was wide. The smaller values of the standard error reassures us that the reported mean of calcconc is a good estimate of the true population mean for this variable. The standard deviation and standard error values for the tissue residue concentration (conc) means for all tests also exhibit similar behavior.

Median Lethal Dose

Table 11 and Table 12 list the median lethal dose, standard error and 95% confidence intervals for the values. A comparison of the median lethal dose values obtained from the pooled data and from data analyzed by colony shows that there is a possible colony influence. Only in one case, colony four of the sodium arsenite tests, was an LD_{50} value (0.54 ug/bee) within the same 95% confidence interval

TABLE 11

MEDIAN LETHAL DOSE
Pooled Data

Chemical Compound	LD_{50}	Standard Error	95% CI
As_2O_3	2.30 ug/bee	0.02	2.30 ± 0.04
$NaAsO_2$	0.54	0.003	0.54 ± 0.01

TABLE 12

MEDIAN LETHAL DOSE
Colony Data

Chemical Compound	Colony	LD_{50}	Standard Error	95% CI
As_2O_3	1	1.55 ug/bee	0.05	1.55 ± 0.10
	2	3.04	0.02	3.04 ± 0.04
	3	1.52	0.16	1.52 ± 0.32
	4	3.02	0.14	3.02 ± 0.26
$NaAsO_2$	1	0.48	0.01	0.48 ± 0.01
	2	0.49	0.004	0.49 ± 0.01
	3	0.33	0.01	0.33 ± 0.02
	4	0.54	0.003	0.54 ± 0.01
	5	0.50	0.005	0.50 ± 0.01

range as the pooled data value (0.54 ug/bee). One main reason for the different colony susceptibilities to arsenic is the colony viability. This viability is related to the age of the colony, the number of bees and brood, food sources, disease, and resistance to environmental stress. The data supports this supposition. Colony four has the highest median lethal dose values (3.02 and 0.54 ug/bee), thus indicating a greater tolerance to arsenic poisoning. This colony was very active, productive and rapidly expanding throughout the summer. On the opposite end of the specturm, colony three, a very small and unproductive colony, had low resistance to arsenic as seen in the low median lethal dose values (1.52 and 0.33 ug/bee). Appendix C contains the mean dose per bee vs probit graphs used to calculate the LD_{50} values.

To determine how real the colony influence is, colonies were organized by test and mean dose levels. Furthermore the sodium arsenite data was plotted by colony on one graph. Table 13 and Figure 8 present the results. The arrangement of colonies by the magnitude of the median lethal dose for arsenic trioxide and sodium arsenite is presented at the bottom of Table 13. If there is a definite, strong colony influence, these colony arrangements should be seen throughout each test. For example, colony four should have consistently the highest mean dose throughout the sodium arsenite tests (Tests 7 - 18).

Table 13 demonstrates that the colony differences are not as great as the LD_{50} values would indicate since the colony arrangements are not consistent. This finding is also borne out by Figure 8

TABLE 13

MEDIAN LETHAL DOSE -
THE ARRANGEMENT OF COLONY DATA BY DOSE

Test	Colony	Mean Dose	Test	Colony	Mean Dose
As_2O_3					
1	2	2.88 ug/bee	4	5	4.83 ug/bee
	4	2.85		4	3.95
	3	1.40		2	3.16
	1	1.02		3	2.78
				1	2.45
2	3	0.29	5	4	0.99
	1	0.26		5	0.80
	2	0.08		1	0.62
	4	0.03		3	0.58
				2	0.40
3	3	0.05	6	4	0.22
	1	0.05		5	0.12
	2	0.05		2	0.08
	4	0.03		1	0.07
				3	0.05
$NaAsO_2$					
7	4	0.11	12	1	0.81
	3	0.11		4	0.53
	1	0.09		3	0.47
	2	0.09		5	0.45
				2	0.41
8	4	0.02	13	4	0.79
	5	0.01		1	0.70
	1	0.01		2	0.64
	3	0.01		5	0.51
	2	0.01		3	0.33
9	All	0.002	14	4	0.72
				1	0.62
				5	0.60
				2	0.48
10	1	0.86	15	4	0.66
	2	0.84		1	0.60
	4	0.83		2	0.53
	3	0.74		5	0.49
	5	0.71			
11	4	0.62	16	1	0.73
	1	0.61		2	0.57
	5	0.59		5	0.56
	2	0.57		4	0.52
	3	0.57			

TABLE 13

Test	Colony	Mean Dose	Test	Colony	Mean Dose
17	1	0.39	18	1	0.28
	4	0.38		5	0.23
	5	0.36		4	0.23
	2	0.28		2	0.21

LD_{50} Order by Colony, As_2O_3

Colony	LD_{50}
2	3.04 ug/bee
4	3.02
1	1.55
3	1.52

LD_{50} Order by Colony, $NaAsO_2$

Colony	LD_{50}
4	0.54 ug/bee
5	0.50
2	0.49
1	0.48
3	0.33

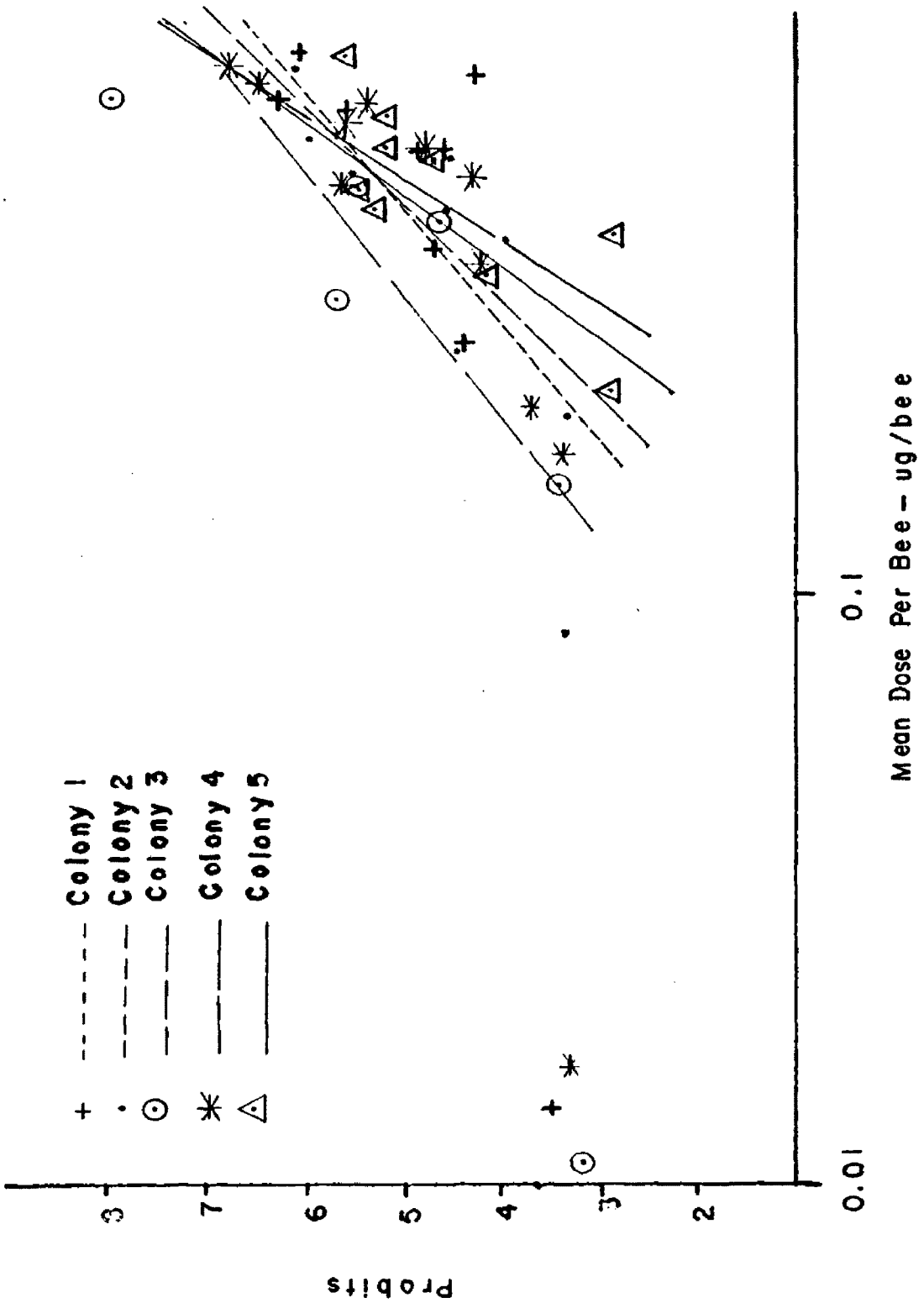


Fig. 8. Median Lethal Dose - Colony Data, $NaAsO_2$.

where the plotted points are all within the same general area. On the other hand, the 95% confidence intervals for the colony LD_{50} values indicate a definite difference between some of the colonies. For the arsenic trioxide tests the colonies are grouped in pairs. Colonies one and three have similar, lower LD_{50} values (1.55 and 1.52 ug/bee) while colonies two and four have high values (3.04 and 3.02 ug/bee). A similar situation is portrayed by the colony LD_{50} values of the sodium arsenite tests. Here colonies one and two are paired with very close LD_{50} values of 0.48 and 0.49 ug/bee. The remaining colonies have separate LD_{50} values which do not have overlapping or similar 95% confidence intervals. Thus even though there is no strong and consistent distinction between colonies there is a suggestion of slight differences in susceptibility.

In the literature, the median lethal dose is often reported in terms of arsenic trioxide. This procedure is misleading since many of the tests were conducted with more soluble forms and then calculated in terms of arsenic trioxide. Thus it is difficult to relate these findings directly to the literature since different forms of the compound have been used. In addition the particle size of the arsenic compounds was not taken into account during this study. As discussed during the literature review, the arsenic compound and particle size do affect the toxicity to honey bees. Although arsenic trioxide is not an arsenate, the only compound within the literature with comparable LD_{50} values to those derived in this study is calcium arsenate. A median lethal dose of 1.3 ug/bee for coarse particles was reported by Bertholf and Pilson (1941) and Wood and Wood (1962)

reported a median lethal dose of 1.4 to 2.1 ug/bee for bumble bees. The oral dose tests conducted by Dr. E.L. Atkins with As_2O_3 had much lower median lethal dose values of 0.07 - 0.104 ug/bee.

Sodium arsenite is much more toxic then arsenic trioxide, as illustrated by median lethal dose values which are as much as ten times smaller then those for arsenic trioxide. Thus even though arsenic trioxide is the source of arsenites, it appears that arsenic in this form is less damaging then the arsenites which are derived from it. This higher toxicity is supported by the literature which states that arsenites are much more active, unstable and soluble then other arsenic compounds. The solubility of sodium arsenite is a definite element in its toxicity. While arsenic trioxide may need to be broken down to a more soluble and toxic form, sodium arsenite is already very soluble and able to attack the insect immediately. The sodium arsenite median lethal dose values, 0.54 - 0.33 ug/bee, compare very closely to reports in the literature. The majority of such reports indicate a median lethal dose of 0.5 ug/bee. In general, poisoning is suspected with 0.07 to 0.6 ug/bee and a lethal dose considered to be 0.12 ug/bee and above (Debackere, 1972). My data supports this statement.

The Effect of Test and Colony Number

The results of the statistical analysis of test and colony effects on the calculated concentration or dose (calcconc) and the concentration of arsenic found during the tissue residue analysis (conc) are presented in Table 14. The null hypothesis tested was whether $u_1 = u_2 = \ldots = u_i$ for the calcconc and conc variables between

TABLE 14

TEST & COLONY EFFECT
Calcconc & Conc

Source of Variation	Sum of Squares	df	Mean Square	F	Significance of F
Calcconc					
As_2O_3					
Main Effects					
Colonyn	3.072	5	0.614	0.438	0.821
Testn	49.826	5	9.965	7.098	0.000
IxT	6.344	21	0.302	0.215	1.000
Explained	63.187	31	2.038	1.452	0.093
Residual	116.520	83	1.404		
$NaAsO_2$					
Main Effects					
Colonyn	0.612	4	0.153	1.974	0.098
Testn	9.135	11	0.830	10.713	0.000
IxT	1.993	38	0.052	0.676	0.928
Explained	11.983	53	0.226	2.916	0.000
Residual	23.178	299	0.078		
Conc					
As_2O_3					
Main Effects					
Colonyn	0.656	5	0.131	0.852	0.517
Testn	2.958	5	0.592	3.843	0.003
IxT	1.730	21	0.082	0.535	0.948
Explained	5.472	31	0.177	1.146	0.304
Residual	13.703	89	0.154		
$NaAsO_2$					
Main Effects					
Colonyn	1.001	4	0.250	1.581	0.179
Testn	15.690	11	1.426	9.011	0.000
IxT	3.675	38	0.097	0.611	0.967
Explained	20.888	53	0.394	2.490	0.000
Residual	47.963	303	0.158		

test and colony groups of bees. A large value of F indicates a high significance (significance of $F \leq 0.05$) while a low value shows a low significance level (significance of $F>0.05$). This is true for all the following F test analyses. A significant F indicates a rejection of the null hypothesis and suggests that the tested groups have different means for the variable of interest. The F values for colony number (Colonyn), range from 0.438 to 1.974, all insignificant. Thus the different colonies do not have significantly different means for the calcconc or conc variables. The colony of the honey bee therefore has no important influence on the value of the dose administered or the amount of arsenic found within the bee. On the other hand, the test number (Testn) is seen to be highly significant with the significance of F being 0.000 to 0.003. This is as it should be since the test number indicates the various arsenic dose levels. We are therefore reassured that honey bees did react differently to the various levels of arsenic.

The two-way interactions analysis (IxT) examines the data after both the colony and test effects have been subtracted out. A high value indicates that what remains is mostly noise. High values, 0.928 - 1.000, are given for the two-way interactions where colonyn and testn are the principle factors accounted for. Thus test and colony are not related and the sum of squares can be placed with the residual to give a better estimate of variance. Testn is the principle element since it constitutes the majority of the explained values for the sum of squares, mean square and F.

Table 15 records the influence of test and colony number on the

TABLE 15

TEST & COLONY EFFECT
Proportion of Dead Bees/Day

PDay	Factor	Sum of Squares	df	Mean Square	F	Significance of F
1	Testn	6.290	23	0.273	9.956	0.000
	Residual	3.846	140	0.027		
	Colonyn	0.865	4	0.216	3.710	0.006
	Residual	9.271	159	0.058		
2	Testn	4.602	23	0.200	18.174	0.000
	Residual	1.542	140	0.011		
	Colonyn	0.216	4	0.054	1.448	0.221
	Residual	5.928	159	0.037		
3	Testn	2.198	23	0.096	25.129	0.000
	Residual	0.532	140	0.004		
	Colonyn	0.044	4	0.011	0.657	0.623
	Residual	2.686	159	0.017		
4	Testn	1.254	23	0.055	17.186	0.000
	Residual	0.444	140	0.003		
	Colonyn	0.005	4	0.001	0.127	0.973
	Residual	1.692	159	0.011		
5	Testn	1.605	23	0.070	16.376	0.000
	Residual	0.597	140	0.004		
	Colonyn	0.035	4	0.009	0.644	0.632
	Residual	2.166	159	0.014		
6	Testn	20.615	23	0.896	81.016	0.000
	Residual	1.549	140	0.011		
	Colonyn	0.340	4	0.085	0.620	0.649
	Residual	21.824	159	0.137		
7	Testn	23.608	23	1.026	172.733	0.000
	Residual	0.832	140	0.006		
	Colonyn	0.133	4	0.033	0.217	0.929
	Residual	24.308	159	0.153		

proportion of dead bees/day (PDay 1-7). Here again the null hypothesis equals $u_1 = u_2 = \ldots = u_i$, but is concerned with the proportion of dead bees/day variable. The proportion of dead bees/day was used to adjust for the different numbers of honey bees used per cage. The dead bee collections of the first day were of honey bees that died due to the transfer process from the field. Subsequent dead bees are assumed to be those poisoned by arsenic. For the first day, the proportion of dead bees/day equals the number of dead honey bees collected on this first day divided by the initial number of bees placed within the cage. The following days are based upon a value of proportion of dead bees/day calculated from the number of dead honey bees collected on the specific day divided by the number of dosed honey bees. As before, the test number indicates the different dose levels and the colony number, the colony number of the honey bees used.

Again the arsenic dose level or test number is the most significant influence on the variation of dead bees/day with significant values of zero. The colony of the honey bees has no effect on the response to arsenic, as indicated by insignificant values of F ranging from 0.217 to 0.448 for PDay 2 through 7. Notice though that the colony number does have a significant effect (0.006 significance level of F) on the mortality response of the first day. Therefore some feature of the colonies appears to influence the tolerance to the transfer process. Colony viability is believed to be the major feature.

The volume of arsenic sugar solution consumed was also modified

by the test number. Table 16 shows a significant F value of 5.667 for testn and an insignificant one of 1.274 for colonyn. Whether there is a difference between the mean volume of arsenic sugar solution consumed between test and colony groups of bees was the null hypothesis tested. As previously hypothesized, a high dose could incapacitate honey bees before they are able to consume much arsenic sugar solution. On the other hand, the colony of the honey bees does not make a difference on the amount of sugar solution consumed. Thus in general, honey bees of different colonies will eat about the same quantity of arsenic sugar solution, receive comparable amounts of arsenic, contain similar concentrations of arsenic in their bodies and will have comparable death rates in response to arsenic.

Collection Day Effect

One question which was investigated was whether there is a difference in the arsenic tissue residue between samples collected on different days from the same bee cage. Dosed samples from Tests 10 through 12 were statistically analyzed. These tests have calculated concentration values of 0.65, 0.46, 0.35 ug/bee and are thus relatively high dose level tests. In addition only samples which were not combined with others to obtain one gram analysis samples were used. Thus samples were unique in terms of cage number, colony number, date and test number.

When the computerized data file was created, the sample dates were designated as the combined number of collection days included within the sample. For instance, if a sample for Test 1 (June 12-17) included dead bee collections from June 13-15 the date in the data

TABLE 16

TEST & COLONY EFFECT
Volume of Arsenic Sugar Solution Consumed

Source of Variation	Sum of Squares	df	Mean Square	F	Significance of F
Main Effects					
Testn	0.055	23	0.002	5.667	0.000
Residual	0.059	140	0.000		
Colonyn	0.004	4	0.001	1.274	0.283
Residual	0.111	159	0.001		

TABLE 17

COLLECTION DAY EFFECT

Source of Variation	Sum of Squares	df	Mean Square	F	Significance of F
Main Effects					
Day 1	0.556	3	0.185	4.119	0.032
Testn	0.268	2	0.134	2.972	0.089
Colonyn	0.216	4	0.054	1.200	0.361
2-way Interactions					
Day 1 Testn	0.167	4	0.042	0.926	0.481
Day 1 Colonyn	0.090	3	0.030	0.664	0.590
Testn Colonyn	0.528	4	0.132	2.933	0.066
Explained	1.941	20	0.097	2.156	0.087
Residual	0.540	12	0.045		

file was recorded as 6 13-15. Other samples which had not been combined were designated by their collection day date. For example 6 12 for June 12. Difficulties occurred in directing the computer to select samples which were only one day collections. Due to these difficulties with the formate of the computerized data file, the results received are general and can only indicate possible trends. In addition there were similar problems in separating out the sacrificed bee samples. Thus at this time there are no reliable results on whether the sacrifice method significantly decreased the arsenic concentration found within these bees.

Table 17 (p. 71) summarizes the statistics of this particular investigation. This analysis tests whether there is a difference between the tissue residue concentration means for bee samples grouped by collection day, test number and colony number. Testn and colonyn describe the test number or dose level and colony number. Day 1 is the specific date the sample was collected. As shown, Day 1 has a significant level of 0.032, suggesting that for the data analyzed there is a difference in the arsenic content of honey bees collected on different days. A possible hypothesis is that the honey bees collected on the first few days contain less arsenic. These honey bees would have had less opportunity to consume much arsenic sugar solution before being poisoned. Those that were more resistant to arsenic would be able to accumulate more of the arsenic before being affected. Another possible explanation is the influence of the sacrifice method. Since this method appeared to desiccate the honey bees some, arsenic could have been lost. Thus the last

day samples would have a different amount of arsenic.

The significance level of F 0.089 in Table 17 implies that the test number does not have a significant influence. This result may be due to the relative close range of the test dose levels where the ranges of the calculated concentrations for each test overlap each other (Test 10: 0.65 ug/bee, Std. dev. 0.346; Test 11: 0.46 ug/bee, Std. dev. 0.256; Test 12: 0.35 ug/bee, Std. dev. 0.229). In addition, the smaller number of cases used in the statistical analysis could have affected the results slightly. As in other instances, the colony of the honey bees with a level of significance of F 0.361 has no noticeable importance in the results.

The Effect of the Number of Honey Bees in the Test Cage

A statistical analysis of the importance of the number of honey bees in the test cage, in conjunction with test number and colony number on the proportion of dead bees/day was conducted. The analysis investigated the null hypothesis that there was no difference between means for the proportion of dead bees/day when the data was grouped by the initial number of honey bees in each cage, number of dosed bees, test number and colony number. The statistics are found in Table 18 and 19. PDay 1-7 are the proportion of dead bees/day as described before. The variables initialb and dosedb represent the initial number of honey bees and the number of dosed honey bees in each cage. The number of dosed bees were those which remained after the die off due to the transfer procedure and which were given the arsenic dose. A study of the sums for each test for initialb and dosedb shows that they varied enormously (Table 20). For the variable

TABLE 18

NUMBER OF HONEY BEES IN TEST CAGE EFFECT
Initial Number of Bees - Testn

PDay	Source of Variation	Sum of Squares	df	Mean Square	F	Significance of F
1	Main Effects					
	Initialb	0.250	4	0.063	2.550	0.045
	Testn	5.599	23	0.243	9.911	0.000
	IxT	1.379	45	0.031	1.248	0.187
	Residual	2.161	88	0.025		
2	Main Effects					
	Initialb	0.019	4	0.005	0.375	0.826
	Testn	4.549	23	0.198	15.774	0.000
	IxT	0.419	45	0.009	0.742	0.864
	Residual	1.103	88	0.013		
3	Main Effects					
	Initialb	0.009	4	0.002	0.575	0.682
	Testn	2.011	23	0.087	23.455	0.000
	IxT	0.194	45	0.004	1.154	0.280
	Residual	0.328	88	0.004		
4	Main Effects					
	Initialb	0.008	4	0.002	0.508	0.730
	Testn	1.190	23	0.052	12.849	0.000
	IxT	0.080	45	0.002	0.441	0.998
	Residual	0.354	88	0.004		
5	Main Effects					
	Initialb	0.010	4	0.002	0.479	0.751
	Testn	1.551	23	0.067	13.506	0.000
	IxT	0.146	45	0.003	0.650	0.943
	Residual	0.440	88	0.005		
6	Main Effects					
	Initialb	0.068	4	0.017	1.257	0.293
	Testn	20.267	23	0.881	65.308	0.000
	IxT	0.293	45	0.007	0.483	0.996
	Residual	1.187	88	0.013		
7	Main Effects					
	Initialb	0.009	4	0.002	0.293	0.882
	Testn	21.809	23	0.948	129.317	0.000
	IxT	0.172	45	0.004	0.520	0.991
	Residual	0.645	88	0.007		

TABLE 18

NUMBER OF HONEY BEES IN TEST CAGE EFFECT
Initial Number of Bees - Colonyn

PDay	Source of Variation	Sum of Squares	df	Mean Square	F	Significance of F
1	Main Effects					
	Initialb	0.660	4	0.165	3.003	0.020
	Colonyn	0.688	4	0.172	3.133	0.017
	IxC	0.655	10	0.065	1.193	0.301
	Residual	7.797	142	0.055		
2	Main Effects					
	Initialb	0.060	4	0.015	0.396	0.811
	Colonyn	0.212	4	0.053	1.412	0.233
	IxC	0.519	10	0.052	1.381	0.195
	Residual	5.340	142	0.038		
3	Main Effects					
	Initialb	0.146	4	0.036	2.207	0.071
	Colonyn	0.016	4	0.004	0.241	0.915
	IxC	0.176	10	0.018	1.069	0.390
	Residual	2.340	142	0.016		
4	Main Effects					
	Initialb	0.059	4	0.015	1.355	0.253
	Colonyn	0.016	4	0.004	0.364	0.834
	IxC	0.058	10	0.006	0.532	0.866
	Residual	1.550	142	0.011		
5	Main Effects					
	Initialb	0.032	4	0.008	0.550	0.700
	Colonyn	0.021	4	0.005	0.370	0.830
	IxC	0.054	10	0.005	0.372	0.957
	Residual	2.062	142	0.015		
6	Main Effects					
	Initialb	0.111	4	0.028	0.192	0.942
	Colonyn	0.413	4	0.103	0.711	0.585
	IxC	0.732	10	0.073	0.504	0.885
	Residual	20.602	142	0.145		
7	Main Effects					
	Initialb	0.700	4	0.175	1.170	0.327
	Colonyn	0.025	4	0.006	0.041	0.997
	IxC	1.364	10	0.136	0.912	0.524
	Residual	21.238	142	0.150		

TABLE 19

NUMBER OF HONEY BEES IN TEST CAGE EFFECT
Number of Dosed Bees - Testn

PDay	Source of Variation	Sum of Squares	df	Mean Square	F	Significance of F
2	Main Effects					
	Dosedb	0.047	5	0.009	0.669	0.648
	Testn	4.370	23	0.190	13.571	0.000
	IxT	0.347	53	0.007	0.467	0.998
	Residual	1.148	82	0.014		
3	Main Effects					
	Dosedb	0.009	5	0.002	0.450	0.812
	Testn	1.965	23	0.085	21.019	0.000
	IxT	0.190	53	0.004	0.882	0.686
	Residual	0.333	82	0.004		
4	Main Effects					
	Dosedb	0.031	5	0.006	1.793	0.123
	Testn	1.218	23	0.053	15.403	0.000
	IxT	0.131	53	0.002	0.721	0.898
	Residual	0.282	82	0.003		
5	Main Effects					
	Dosedb	0.032	5	0.006	1.328	0.261
	Testn	1.575	23	0.068	14.103	0.000
	IxT	0.166	53	0.003	0.645	0.955
	Residual	0.398	82	0.005		
6	Main Effects					
	Dosedb	0.073	5	0.015	1.152	0.340
	Testn	18.648	23	0.811	64.033	0.000
	IxT	0.438	53	0.008	0.652	0.951
	Residual	1.038	82	0.013		
7	Main Effects					
	Dosedb	0.033	5	0.007	1.200	0.317
	Testn	20.271	23	0.881	159.385	0.000
	IxT	0.345	53	0.007	1.178	0.249
	Residual	0.453	82	0.006		

TABLE 19

NUMBER OF HONEY BEES IN TEST CAGE EFFECT
Number of Dosed Bees - Colonyn

PDay	Source of Variation	Sum of Squares	df	Mean Square	F	Significance of F
2	Main Effects					
	Dosedb	0.307	5	0.061	1.809	0.115
	Colonyn	0.244	4	0.061	1.794	0.133
	IxC	0.902	15	0.060	1.772	0.044
	Residual	4.719	139	0.034		
3	Main Effects					
	Dosedb	0.243	5	0.049	3.239	0.008
	Colonyn	0.045	4	0.011	0.753	0.557
	IxC	0.360	15	0.024	1.600	0.081
	Residual	2.083	139	0.015		
4	Main Effects					
	Dosedb	0.067	5	0.013	1.280	0.276
	Colonyn	0.006	4	0.001	0.140	0.967
	IxC	0.163	15	0.011	1.034	0.424
	Residual	1.462	139	0.011		
5	Main Effects					
	Dosedb	0.064	5	0.013	0.910	0.476
	Colonyn	0.037	4	0.009	0.662	0.619
	IxC	0.145	15	0.010	0.685	0.796
	Residual	1.958	139	0.014		
6	Main Effects					
	Dosedb	2.111	5	0.422	3.403	0.006
	Colonyn	0.411	4	0.103	0.829	0.509
	IxC	2.468	15	0.165	1.326	0.195
	Residual	17.245	139	0.124		
7	Main Effects					
	Dosedb	3.438	5	0.688	5.303	0.000
	Colonyn	0.200	4	0.050	0.385	0.819
	IxC	2.848	15	0.190	1.464	0.127
	Residual	18.022	139	0.130		

TABLE 20

GENERAL STATISTICS -
NUMBER OF BEES PER CAGE AND NUMBER OF DOSED BEES

Test	Actual Dose	Initialb	Dosedb
As_2O_3			
1	0.22 ug/bee	440	189
2	0.09	338	166
3	0.09	416	180
TC1-3	0.00	274	171
4	2.28	2297	1297
5	0.33	2540	1027
6	0.04	1845	1000
TC4-6	0.00	929	288
$NaAsO_2$			
7	0.05	1263	787
8	0.01	1222	743
9	0.001	960	652
TC7-9	0.00	636	391
10	0.65	2175	1808
11	0.46	2329	1997
12	0.35	2980	2622
TC10-12	0.00	903	791
13	0.49	1909	1546
14	0.47	1700	1367
15	0.46	2136	1625
TC13-15	0.00	846	695
16	0.34	1398	421
17	0.19	1263	372
18	0.12	1471	488
TC16-18	0.00	817	350

initialb the range between tests was from 274 to 2980 and for dosedb 166 to 2622. The proportion of dead bees/day was used to eliminate any effect due to this variability.

As indicated in Table 18, the number of honey bees placed in the cage and their colony origin changed the response to the first day of the test. Both of these variables have significant values of F; 2.550, 3.003 for initialb and 3.133 for colonyn. Furthermore Table 8 (p. 51) depicts a high mortality for this first day. It is hypothesized that this response is to the transfer procedure. Since the number of honey bees placed in the cages is also significant, it can be hypothesized that crowding may increase or decrease the ability to withstand shock. PDay 1 also has a significant value of F, 9.911, for testn. Since arsenic was not administered at this time, the significant difference between groups could be due to the time of collection for each test. For instance honey bees collected for Tests 10-12 during the middle of the summer were younger and more resistant to shock then those collected for tests conducted at the summer's end.

With significance levels of zero for F, the remainder of the PDay statistics in Table 18 show that the test number is again the major element of mortality. As indicated by insignificant values of F, neither the colony of the honey bees nor the number of bees within the cage had a major role in this mortality response.

The statistics for dosedb in conjunction with testn, colonyn, and PDay are in Table 19. The same conclusions and inferences can be drawn as with the initialb statistics. The arsenic dose level

is the major factor of mortality and the colony of the honey bees and number of dosed bees are unimportant. There are a few notable exceptions where the number of dosed honey bees appears to be significant. For instance PDay 6 has an F value of 3.403 for dosedb in conjunction with colonyn which has a significance level of 0.006, a significant value. These cases only appear when dosedb and colonyn are used as the main effects. Possible explanations are that the influence of the test differences is being indirectly shown, that there is some small but infrequent reaction to the number of honey bees in the cage, and that these values are normal random errors.

Throughout the statistical analyses, the colony of the honey bees has been shown to be unimportant in relation to the amount of arsenic residue found in the bees, the calculated arsenic dose administered, proportion of dead bees/day, and the volume of arsenic sugar solution consumed. Yet the median lethal dose data demonstrate a colony factor. One possible hypothesis is that honey bees of different colonies are susceptible to different levels of arsenic. Yet when they do respond, the level of response is influenced more by a reaction to the poison then by differences between colonies. Therefore the mortality response to lethal doses of arsenic will be similar between colonies although each colony may be more susceptible to different dose levels.

Arsenic Tissue Residue

General statistics for the concentration of arsenic found in the honey bees during the tissue residue analysis are summarized in Table 21. This variable has been labeled conc. The variables

TABLE 21

ARSENIC TISSUE RESIDUE

Test[1]	Mean Calcconc[2]	Mean Conc[3]	% Difference[3]	Standard Error	Standard Deviation
As_2O_3					
1	0.22	0.50	56	0.22	0.71
2	0.09	0.18	50	0.06	0.20
3	0.09	0.07	32	0.02	0.07
4	2.28	0.65	249	0.08	0.45
5	0.33	0.15	120	0.04	0.15
6	0.04	0.06	41	0.02	0.08
$NaAsO_2$					
7	0.05	0.08	37	0.02	0.06
8	0.01	0.05	89	0.02	0.06
9	0.001	0.04	98	0.01	0.05
10	0.65	0.76	14	0.07	0.41
11	0.46	0.57	18	0.07	0.40
12	0.35	0.62	44	0.07	0.38
13	0.49	0.62	21	0.06	0.36
14	0.47	0.58	18	0.08	0.44
15	0.46	0.80	42	0.07	0.41
16	0.34	0.60	43	0.13	0.57
17	0.19	0.39	52	0.10	0.38
18	0.12	0.26	52	0.06	0.23
TC 1-3	0.00	0.02		0.01	0.02
CC 1-3		0.20		0.06	0.14
TC 4-6		0.03		0.004	0.01
CC 4-6		0.02		0.004	0.01
TC 7-9		0.04		0.02	0.05
CC 7-9		0.03		0.01	0.03
TC10-12		0.04		0.02	0.05
CC10-12		0.03		0.03	0.08
TC13-15		0.05		0.02	0.05
CC13-15		0.06		0.02	0.06
TC16-18		0.02		0.003	0.01
CC16-18		0.02		0.01	0.01

[1]TC - Test Control , CC - Colony Control

[2]Mean calculated concentration of arsenic administered, ug/bee.

[3]Mean tissue residue levels of arsenic, ug/bee.

[4]% difference between mean calcconc and mean conc.

TC # to # and CC # to # are the test and colony control samples for the designated test numbers. The majority of the % differences are fairly high and there is no apparent close correspondence. This lack of correspondence may be due to the fact that the means of the calcconc and conc variables are being compared here. This relationship was studied in more detail and will be discussed later.

Despite the apparent lack of correlation, the statistics do illustrate a number of trends. When organized by dose level, the % difference values increase as the calculated doses decrease. As usual, the arsenic trioxide samples are variable and definitely do not appear to correspond well with the calcconc values. The sodium arsenite samples are more reliable and have tissue residue concentrations consistently higher then the arsenic administered. The samples were not washed which could have contributed to these higher tissue residue concentrations. One can also hypothesize that the chemical analysis of the honey bees is a more accurate method of determining the arsenic actually received by them. Furthermore honey bees are known to concentrate certain impurities in their body tissues (Bromenshenk and Gordon, 1978), thus the higher tissue residue may be due to such a mechanism. The conc values also follow the trend of high to low values which in general match the given arsenic dose levels. Again the middle range of concentrations tend to blend together so that some higher doses have lower tissue residue values then related but lower doses. For instance, Test 16 has a tissue residue level of 0.600 ug/bee although it is of a lower calculated dose then Test 11 which has a tissue residue level of

0.57 ug/bee.

The control samples show uniformly low arsenic concentrations with a mean of 0.03 ug/bee. The colony control samples for Tests 1 to 3 are the only exceptions to this trend, having a mean tissue residue concentration value of 0.20 ug/bee. Possible explanations are that this value is a normal random error or that some contamination did occur.

The Relationship Between the Calculated Actual Dose (Calcconc) and Tissue Residue (Conc)

The SPSS programs Scattergram, Pearson Corr and Regression were used to analyze the relationship between the calculated concentration of arsenic actually administered to the honey bees (calcconc) and the arsenic concentration found in the bee tissue (conc). All the programs produced similar results. Figures 9 and 10 present the results of the Scattergram where conc is the dependent variable (y) and calcconc is the independent one (x). Scattergram is presented because it includes correlation coefficients and regression equations in addition to the standard statistics obtained in the other SPSS programs.

There is a very significant and positive correlation between the two variables with a correlation coefficient of 0.8923 for arsenic trioxide and 0.8606 for sodium arsenite. The high r value for arsenic trioxide may be slightly higher then in actuality. Looking at Figure 9, it appears that there are two distinct groups of data. Including both these data groups in the same regression analysis will increase the correlation coefficient value due to the assumed regression line between them. For arsenic trioxide 80% and for

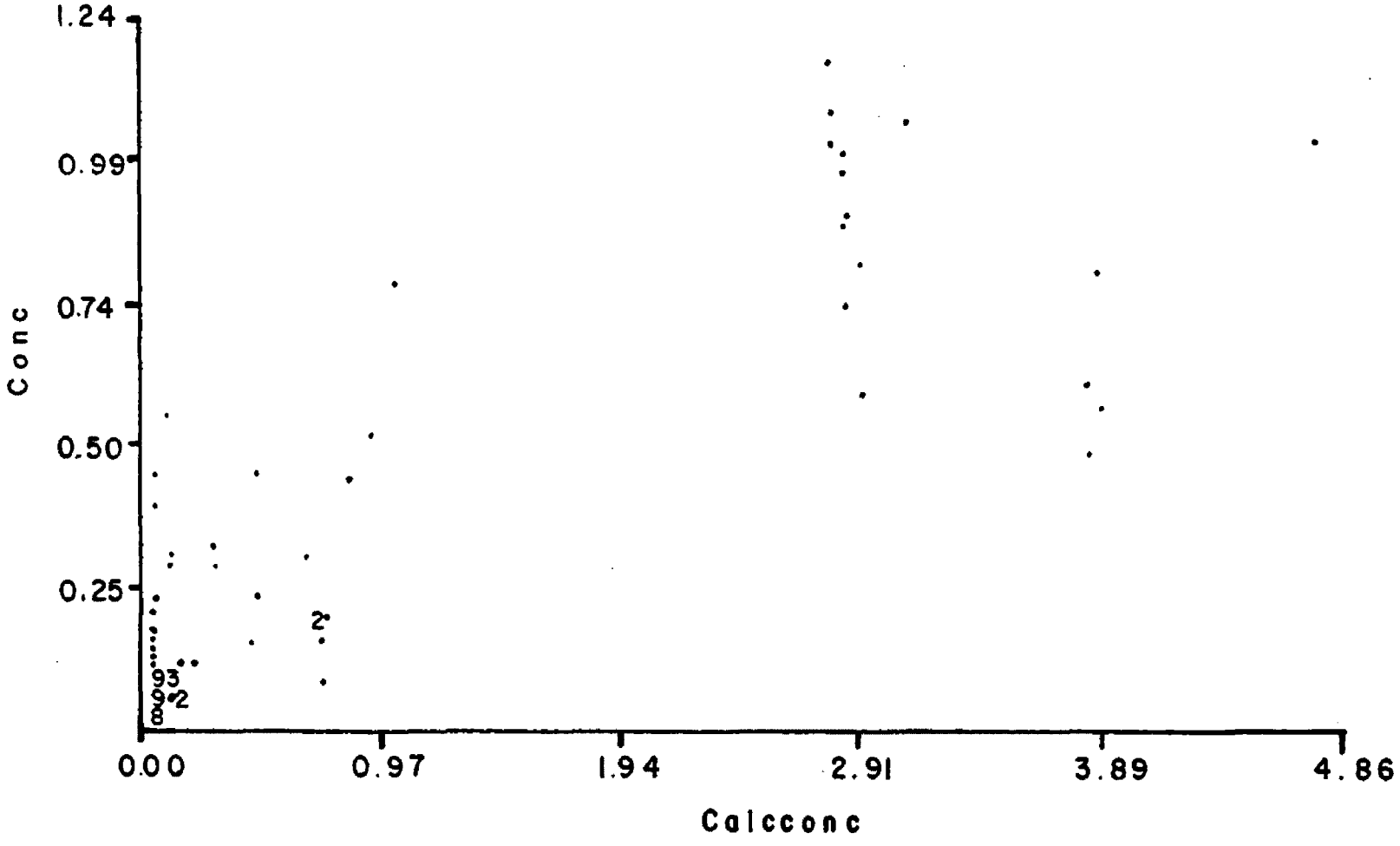


Fig. 9. Scattergram of (down) Conc - concentration calculated from chemical analysis. (across) Calcconc - concentration calculated from the arsenic liquid consumed. As_2O_3.

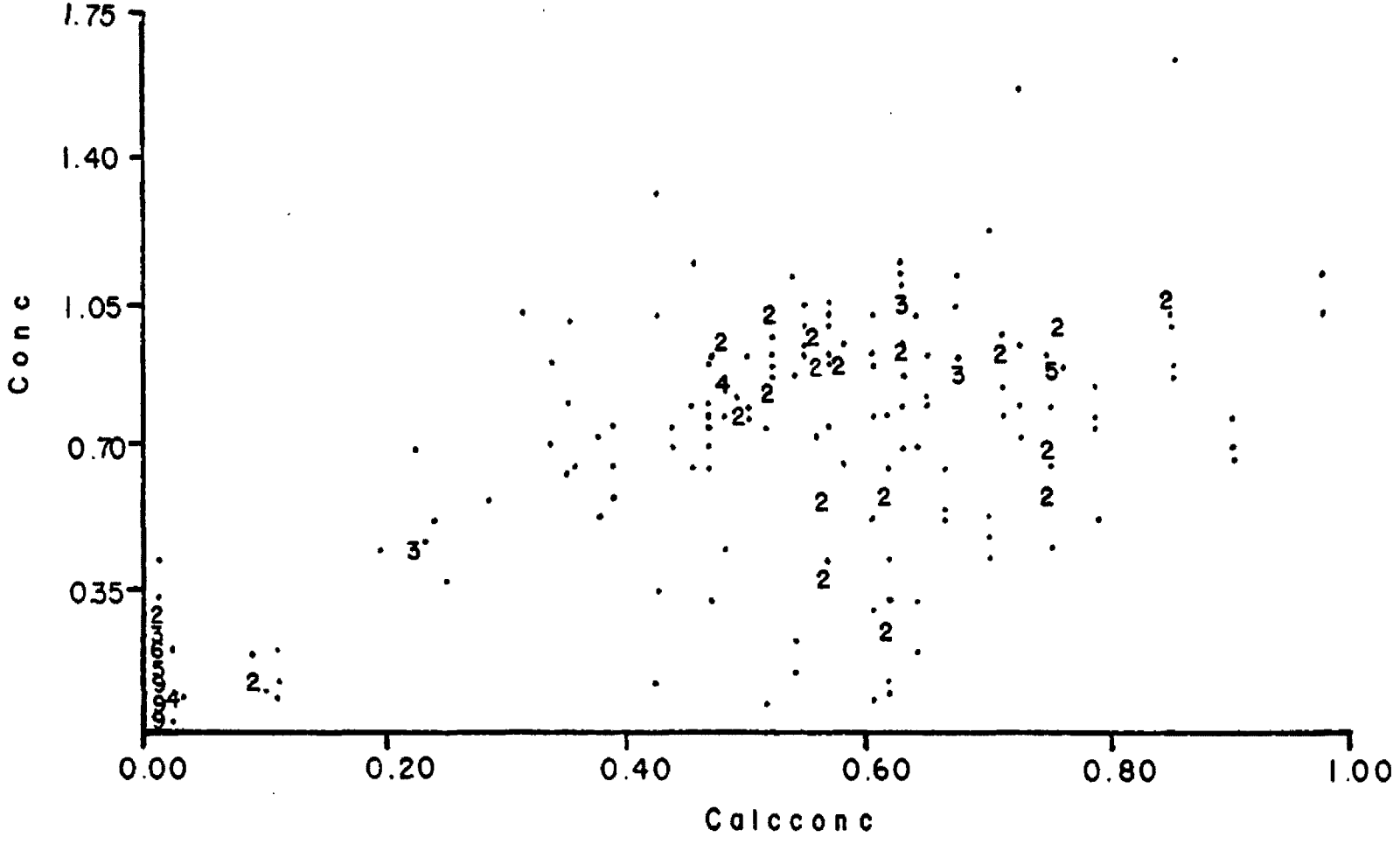


Fig. 10. Scattergram of (down) Conc - concentration calculated from chemical analysis. (across) Calcconc - concentration calculated from the arsenic liquid consumed. $NaAsO_2$.

sodium arsenite 74% of the tissue residue level is explained by the calculated concentration of arsenic administered to the honey bees. The correlation equations, y = 0.07559 + 0.24642x for arsenic trioxide and y = 0.7283 + 1.19179x for sodium arsenite, indicate that y, the tissue residue level, is always slightly higher then the calculated dose, x. Thus even though calcconc and conc do not appear to correspond when looking at their mean values for each test, in actuality they are highly correlated.

Scattergrams were also run for the opposite situation where calcconc was the dependent variable and conc the independent one. Here too there is a very positive and significant correlation. Therefore the calculated concentration of the arsenic dose administered in oral dose tests is a reliable estimate of the tissue residue levels within the dosed bees. The opposite is also true, where the tissue residue of arsenic in caged laboratory dosed bees is a good indicator of the arsenic dose. The correlation equations can be used to relate the two variables to each other.

Covariate analyses were done, alternating calcconc and conc as the covariates. Table 22 gives these results. A covariate is defined as a metric factor which is subtracted from the dependent variable before and analysis of variance is applied. The program also runs a simple regression analysis on the covariate and dependent variable. The results of the covariate analysis show a significance level of zero for the F value of the covariate. This indicates that the calculated arsenic dose and tissue residue arsenic concentration are closely related. The analysis of variance on the residue after

TABLE 22

COVARIATE ANALYSIS
Calcconc & Conc

Source of Variation	Sum of Squares	df	Mean Square	F	Significance of F
As_2O_3					
Calcconc					
Covariate Conc	143.778	1	143.778	486.360	0.000
Main Effect Colonyn	4.561	6	0.760	2.571	0.023
Residual	32.223	109	0.296		
Conc					
Covariate Calcconc	10.964	1	10.964	495.528	0.000
Main Effect Colonyn	0.393	6	0.066	2.962	0.010
Residual	2.412	109	0.022		
$NaAsO_2$					
Calcconc					
Covariate Conc	25.976	1	25.976	1069.658	0.000
Main Effect Colonyn	0.698	4	0.174	7.182	0.000
Residual	8.402	346	0.024		
Conc					
Covariate Calcconc	49.821	1	49.821	1071.931	0.000
Main Effect Colonyn	1.372	4	0.343	7.381	0.000
Residual	16.081	346	0.046		

the covariate has been subtracted, with colonyn as the main effect, generated significant values of F ranging from 2.571 to 7.381. All these F values have significance levels of 0.023 or zero. The colony of the honey bees therefore has some influence on the variation observed. Previous studies have shown that this colony effect becomes unimportant when the dose level is considered. The most probable explanation for this significance of the colony is that the test effect is being picked up as a difference between colony groups.

CHAPTER V

SUMMARY AND CONCLUSIONS

Digestion method #1 for honey bees is shown to be much more thorough and reliable then the second method described. Oven dried samples are slightly lower in arsenic content than freeze dried samples but prove to be more consistent and convenient.

An unknown residue appeared in the digestion samples. Various tests indicate that this residue is nonorganic, not potassium and fairly insoluble in distilled water. Despite the unknown residue, a high correlation between the known arsenic content of the samples and the arsenic calculated from the chemical analysis was established. Other investigations demonstrated that the chemical procedure and technique were highly reliable.

Mortality response graphs, plotting % dosed dead bees/day either separately or accumulatively by collection day, illustrate definite trends in response to arsenic poisoning. Specific graph configurations can be matched with high, medium and low dose levels. Arsenic trioxide appears to be much more variable then sodium arsenite -- a variability which is noticeable throughout the data analysis. In addition, a difference in response is seen between two separate dose periods and one dose period where the level of dose is supposedly the same.

The above trends also are demonstrated by the number of dying bees and the general statistics of the daily mortality rate of the honey bees. The number of dying honey bees itself is an indication of

the severity of the arsenic poisoning. A high arsenic dose induces a high number of dying bees.

The calculated concentration of the arsenic actually given the honey bees did not agree with the theoretical dose. It has been shown that the assumption of 0.2 ml of arsenic sugar solution per bee per day which was the basis of the theoretical dose calculations is far too high, the actual value being approximately 0.03 ml per bee per day. Absorbance of arsenic onto the plastic feeding vials or loss of arsenic during the 24 hour dose period prove not to be significant. Furthermore the analysis of the stock solutions did not show any major loss of arsenic due to age or absorbance onto the glassware.

Although pairs of colonies had similar LD_{50} values, (colonies 1 & 3, 2 & 4 for As_2O_3 and colonies 1 & 2 for $NaAsO_2$), the median lethal dose data reveals a colony response to arsenic. Each colony was susceptible to a different arsenic level. Arsenic trioxide was much less toxic and consistent then sodium arsenite. The median lethal dose for sodium arsenite results correspond well to the values reported in the literature. The general response in terms of mortality, tissue residue of arsenic and to other experimental factors was the same regardless of the identity of the colony of the honey bees. The only other time the colony of the honey bees was seen to be an influencing element was in the response and resistence to the transfer procedure from the field to the test cages.

The test number or dose level of arsenic is the major factor influencing the variation between groups of honey bees in relation to

the calculated concentration of arsenic administered, the concentration of the tissue residue, proportion of dead bees/day and the volume of arsenic sugar solution consumed. As indicated, this result is logical and reassuring since the dose level was the major influencing element of interest. Furthermore, other investigations have proved that the collection day and the number of honey bees in the test cages have very little significant influence on the response of the honey bees in relation to the arsenic dose level. Thus it can be concluded that the arsenic poisoning was the main component of the oral dose tests and tissue residue results.

The chemical analysis results for the sodium arsenite samples show a higher level of arsenic then believed to be administered. The samples do correspond in magnitude to the dose. In addition the arsenic background level of honey bees is proved to be about 0.03 ug/bee.

This study demonstrates a definite significant positive correlation between the orally administered dose and tissue residue of dosed honey bees. These results support the literature which indicates that arsenic is lethal mainly as an ingested stomach poison (George Grant Ballantine, d/b/a Cloverdale Apiaries, Plaintiff vs Anaconda Company, Defendant, In District Court of the Fifth Judicial District of the State of Montana, In and For the County of Jefferson, 1976). As such, the tissue residue concentration should reflect the lethal dose of arsenic. Thus a correlation between oral dose toxicity and tissue residue levels has been confirmed in the laboratory. The correlation equations, $y = 0.07559 + 0.24642x$ for arsenic trioxide

and $y = 0.7283 + 1.19179x$ for sodium arsenite can be used to relate oral dose toxicity or the calculated dose (x) to tissue residue levels (y). There are still many other factors such as defecation in flight, colony viability and environmental stress which must be investigated before the findings of this study can be related to a field situation. Therefore field confirmation of the relationship between oral dose toxicity and tissue residue levels is necessary and highly recommended.

LITERATURE CITED

Anderson,L.D. and E.L. Atkins, Jr. 1968. Pesticides Usage in Relation to Beekeeping. Annu. Rev. Entomol. 13:213-238.

Batson,H.C. 1960. An Introduction to Statistics in the Medical Sciences. Burgess Publishing Co. Minn.

Beard,R.L. 1949. Species - Specificity of Toxicants as Related to Route of Administration. Jour. of Econ. Ent. 42(2):292-300.

Behan,M.J. and T. Kinraide. 1970. Rapid Wet Ash Digestion of Coniferous Foliage for Analysis of Potassium, Phosphorus, Calcium and Magnesium. Bulletin 39. Montana Forest and Conservation Experiment Station, School of Forestry, University of Montana, Missoula, Montana.

Bertholf,L.M. and J.E. Pilson. 1941. Studies on Toxicity to Honeybees of Acid Lead Arsenate, Calcium Arsenate, Phenothiazine and Cryolite. Jour. of Econ. Ent. 34(1):24-32.

Bromenshenk,J.J. 1978. Yet Another Job for Busy Bees. The Sciences. 18(6):12-15.

Bromenshenk,J.J. 1978. Chapter 6: Honey Bees and Other Insects as Indicators of Pollution Impact from Coalstrip. In: E.M. Preston and T.L. Gullett. Eds. The Bioenvironmental Impact of a Coal-Fired Power Plant. EPA-600/3-79-044. Corvallis Environmental Research Laboratory, Office of Research and Development. U.S. Environmental Protection Agency , Corvallis, Oregon.

Bromenshenk,J.J. and C.C. Gordon. 1978. Terrestrial Insects Sense Air Pollutants. In: Proceedings of the Fourth Joint Conference on Sensing of Environmental Pollutants, November 6-11, 1977. New Orleans, Louisiana. American Chemical Society, Washington, D.C. pp 66-70.

Bromenshenk,J.J. and J.B. States. eds. 1980. Biological Monitoring of Terrestrial Ecosystems Using Honey Bees and Earthworms - A Workshop Summary and Panel Reports. Corvallis Environmental Research Laboratory. Office of Research and Development. U.S. Environmental Protection Agency. Corvallis, Oregon. 48 pp.

Brown,A.W.A. 1951. Insect Control by Chemicals. John Wiley & Sons, Inc. New York. p 31-54, 174-278.

Cook,F.C. and N.E. McIndoo. 1923. Chemical, Physical and Insecticidal Properties of Arsenicals. USDA Bulletin 1147. 57 pp.

Debackere,N. 1972. Industrial Air Pollution and Apiculture. Vlaams Imkersblad. 2(6):145-155.

Eckert,J.E. 1944. The Beekeeping Industry and Insecticides. Gleanings in Bee Culture. 72(2):1-4.

Eckert,J.E. and H.W. Allinger. 1935. Airplane Dusting and Its Relation to Beekeeping. Jour. of Econ. Ent. 28:590-597.

Eckert,J.E. and H.W. Allinger. 1936. Relation of Airplane Dusting to Beekeeping. Jour. of Econ. Ent. 29(5):885-893.

Ellisor,L.O. and C.R. Blair. 1940. Effect of Temperature Upon the Toxicity of Stomach Poisons. Jour of Econ. Ent. 33(5):760-762.

Ferencik,M. 1961. Industrial Poisoning of Bees and Its Diagnosis. Vet. Casopia. 10(4):377-382.

Harkins,W.D. and R.E. Swain. 1907. The Determination of Arsenic and Other Solid Constituents of Smelter Smoke, with a Study of the Effects of High Stacks and Large Condensing Flues. Jour. of the American Chemical Society. 29(4):970-998.

Hoskins,W.M. and A.S. Harrison. 1934. The Buffering Power of the Contents of the Ventriculus of the Honeybee and its Effect Upon the Toxicity of Arsenic. Jour. of Econ. Ent. 28:924-942.

Knowlton,G.F., L.W. Jones, and W.P. Nye. 1948. An Arsenic Survey of Utah. Gleanings in Bee Culture. 76(1):8-9.

Knowlton,G.F., A.P. Sturtevant, and C.J. Sorenson. 1950. Adult Honeybee Losses in Utah as Related to Arsenic Poisoning. Bulletin 340. Experiment Station, Utah State Agricultural College. 30 pp.

Lillie,R.J. 1972. Chapter 6: Arsenic. In: Air Pollutants Affecting the Performance of Domestic Animals: A Literature Review. Revised. Agric. Handbook No. 380. Agric. Research Service, USDA. p 20-23.

Luepke,N.-P. Ed. 1978. Monitoring Environmental Materials and Specimen Banking. Proceedings of the International Workshop, Berlin (West) 23-28 Oct 1978. Martinus Nijhoff Publishers. 591 pp.

McGregor,S.E. 1976. Insect Pollination of Cultivated Crop Plants. Agriculture Handbook No. 496. USDA Agricultural Research Service, Washington,D.C. 411 pp.

McIndoo,N.E. and G.S. Demuth. 1926. Effect on Honeybees of Spraying Fruit Trees with Arsenicals. USDA Bulletin 1364. 32 pp.

Milum,V.G. 1930. Further Notes on Spray Poisoning. Amer. Bee. Jour. 70(4):176-178.

Montana Department of Agriculture and Montana Crop and Livestock Reporting Service. 1978. Montana Agricultural Statistics. Vol XVII. County Statistics 1976 and 1977. Federal Building and U.S. Courthouse. P.O. Box 4369. Helena, Montana.

Root,E.R. 1907. Destruction of Bees by Smelter Smoke: A Settlement with the Smelters by Which the Suffering Beekeepers were Awarded $60,000 Damages. Gleanings in Bee Culture. 35(9): 616-617.

Root,E.R. and M.J. Deyell. 1941. Destruction of Bees by Smelter Gases. Gleanings in Bee Culture. 10:638.

Rousseau,par (M.) and M^{ME} Pangaud. 1959. Lethal Dose of Arsenic and Digestion of the Bee. Bulletin Apicole. p.27.

Shaw,F.R. 1941. Bee Poisoning: A Review of the More Important Literature. Jour. of Econ. Ent. 34(1):16-21.

Shepard,H.H. 1939. The Chemistry and Toxicology of Insecticides. Chapter 5: Stomach Poisons. Burgess Publishing Co. Minneapolis, Minn. p 47-109.

Siemer,D.D., P. Koteel, and V. Jarwala. 1978. Optimization of Arsine Generation in Atomic Absorption Arsenic Determiniations. Anal. Chem. 48(6):836-841.

Sonnet,P.E., T.L. Lye, and R.A. Sackett. 1978. Effects of Selected Herbicides on the Toxicity of Several Insecticides to Honey Bees. Environmental Entomology. 7(2):254-256.

Sturtevant,A.P., G.F. Knowlton,J.D. Hitchcock, G.H. Vansell, E.C. Holst, and W.P. Nye. 1941. A Report of Investigations of the Extent and Causes of Heavy Losses of Adult Honeybees in Utah. Number E-545. Division of Bee Culture, Bureau of Entomology and Plant Quarantine, USDA and Utah Agricultural Experiment Station. 18 pp.

Svoboda,J. 1960. XVII International Beekeeping Congress. Poisoning of Bees from Industrial Plant Exhalation. Bienenforschungsanstalt Dol, p. Libschitz an der Moldau. Czechoslovakia. p 113.

Swain,R.E. and W.D. Harkins. 1908. Arsenic in Vegetation Exposed to Smelter Smoke. Jour. of the American Chemical Society. 30(6):915-928.

Tietz,H.M. 1924. The Solubility of Arsenate of Lead in the Digestive Fluids of the Honeybee. Jour. of Econ. Ent. 17:471-477.

Toshkov,A.S., M.M. Shabanov, and N.E. Ibishimov. 1974. Attempts to Use Bees to Prove Impurities in the Environment. Air Pollution Indicators. Comptes rendus de l'Academie bulgare des sciences. 27(5):699-702.

Troop,J. 1918. Blossom Spraying and Bee Poisoning. Jour. of Econ. Ent. 11:433.

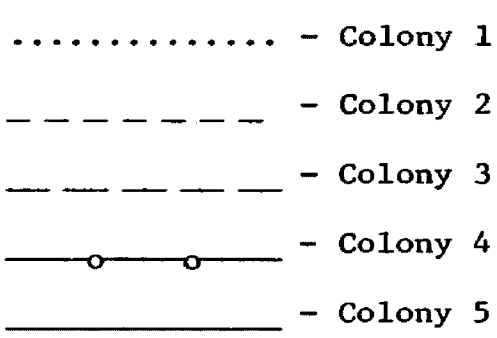
Winski,T.M. 1974. If You Don't Have Enough Woes, Try Fretting About Bees: They are Eating Pesticides and Dying, Hurting Yields of Many Key Food Crops. Wall Street Journal 11/7/74. p 1.

Wood,G.W. and F.A. Wood. 1962. Mortality of Bumble Bees in Commercial Low-Bush Blueberry Fields Dusted with Calcium Arsenate. Jour. of Econ. Ent. 55(4):537-539.

APPENDIX A

% DOSED DEAD BEES/DAY MORTALITY RESPONSE GRAPHS

Legend:



Use this legend for Appendix A and B.

Each line of each graph represents a separate test cage.

The mortality for the last collection day represents sacrificed honey bees.

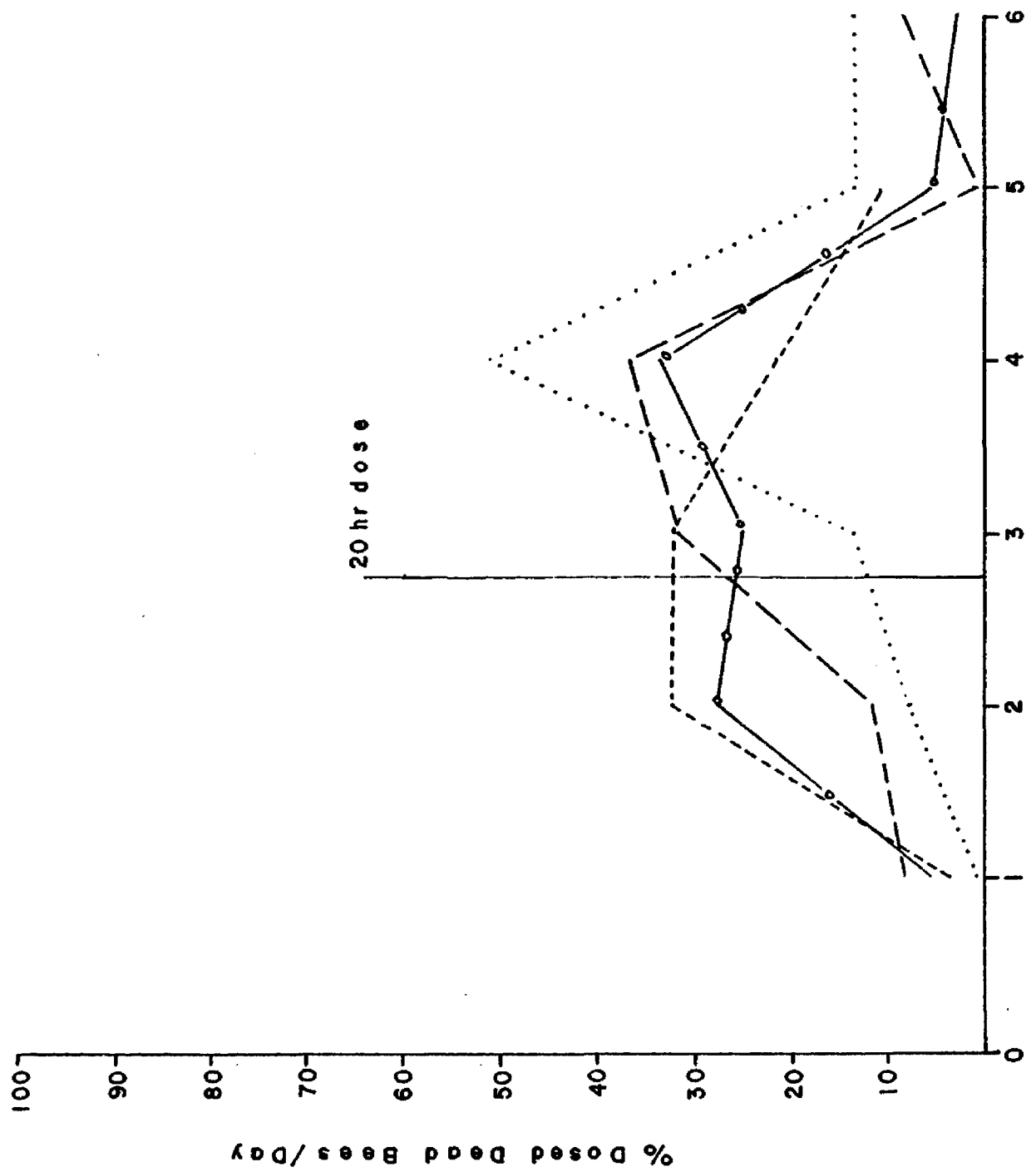


Fig. 1. Test 1 - As_2O_3. Theoretical Dose: 4 hr - 0.5 ug/bee, 24 hr - 3.0 ug/bee. Actual Dose (Mean Calcconc): 24 hr - 0.22 ug/bee.

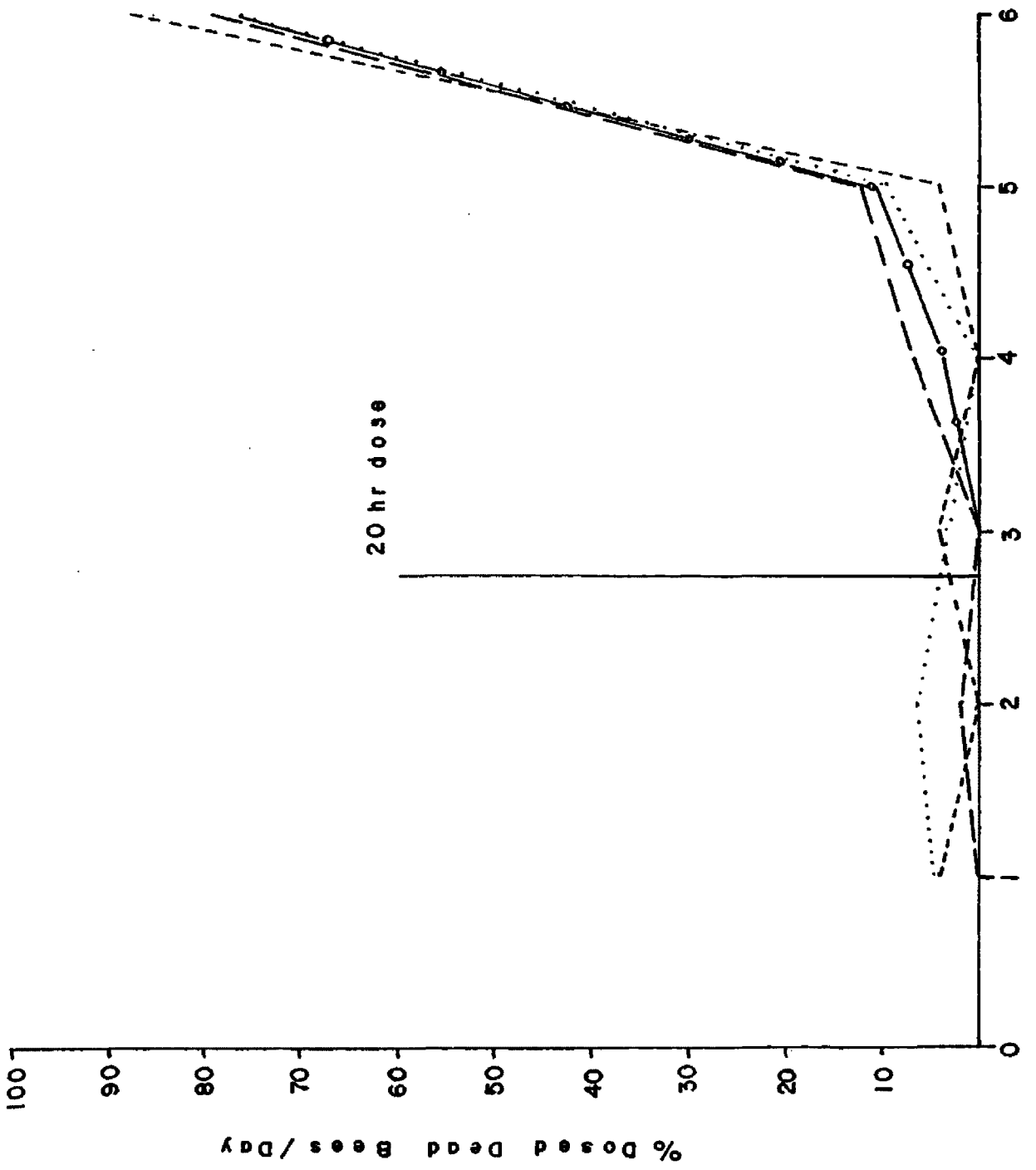


Fig. 2. Test 2 - As_2O_3. Theoretical Dose: 4 hr - 0.07 ug/bee, 24 hr - 0.42 ug/bee. Actual Dose (Mean Calcconc): 24 hr - 0.087 ug/bee.

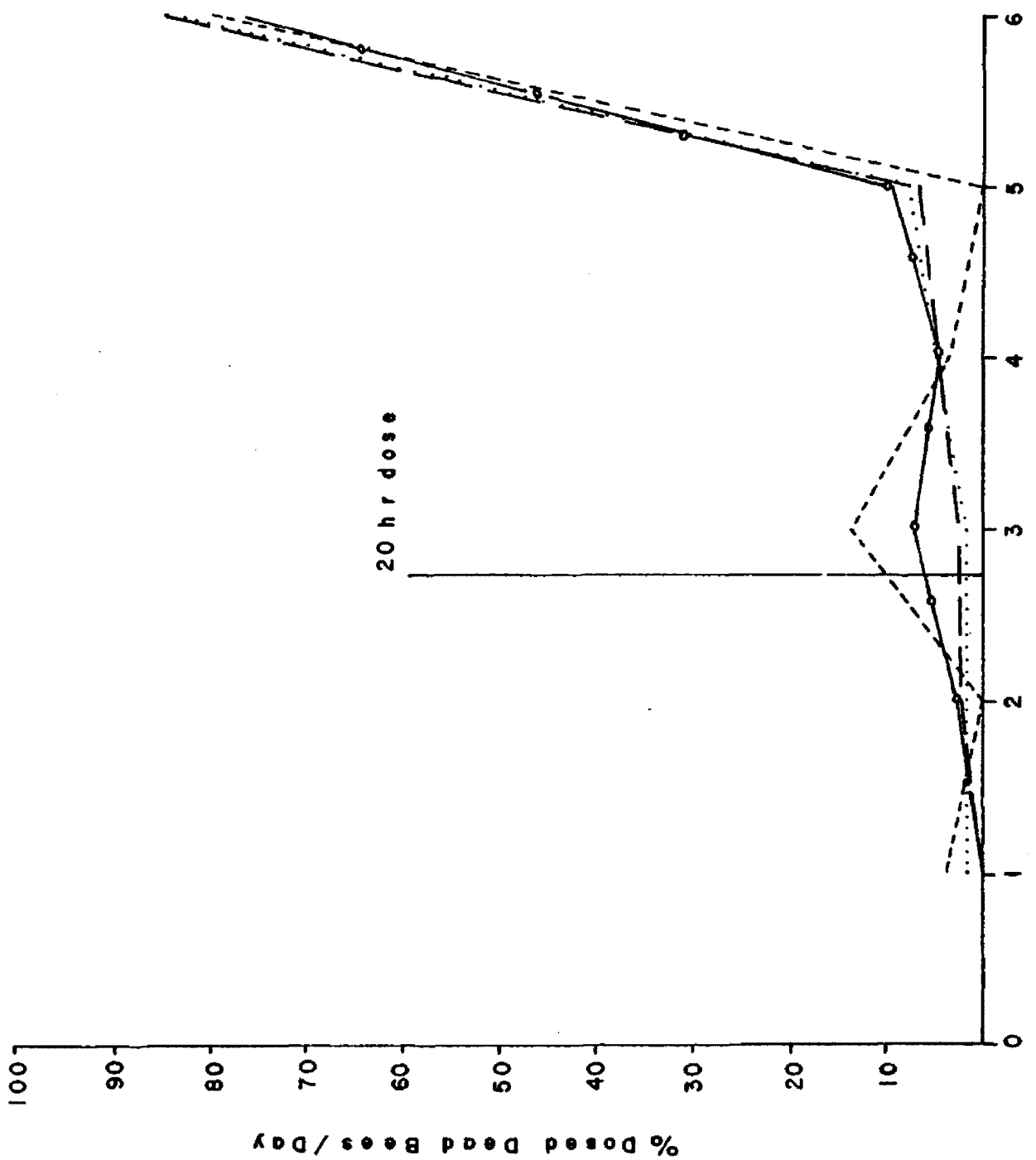


Fig. 3. Test 3 - As_2O_3. Theoretical Dose: 4 hr - 0.01 ug/bee, 24 hr - 0.06 ug/bee. Actual Dose (Mean Calcconc): 24 hr - 0.09 ug/bee.

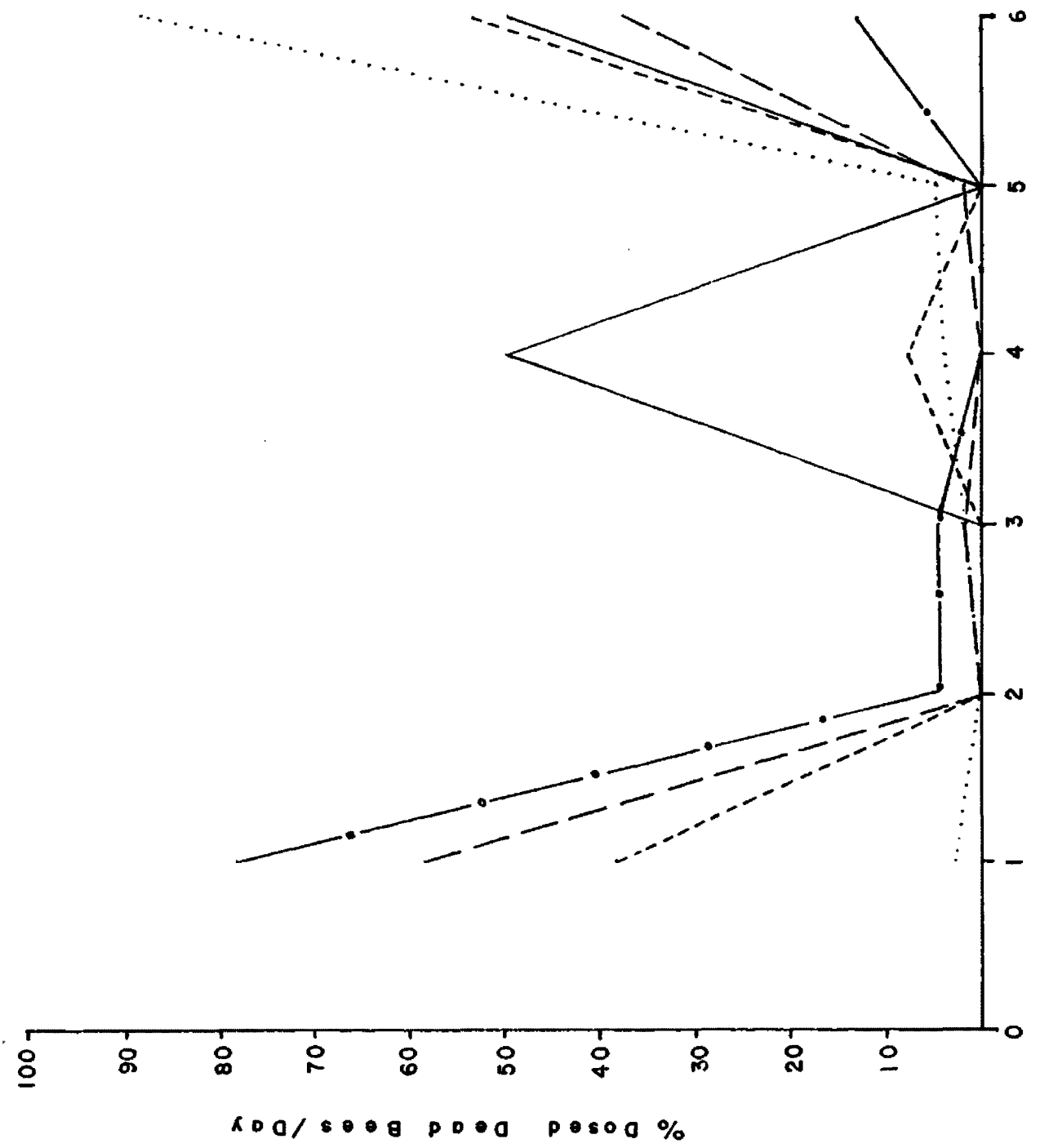


Fig. 4. Control: Tests 1-3. Theoretical Dose: 0.0 ug/bee.

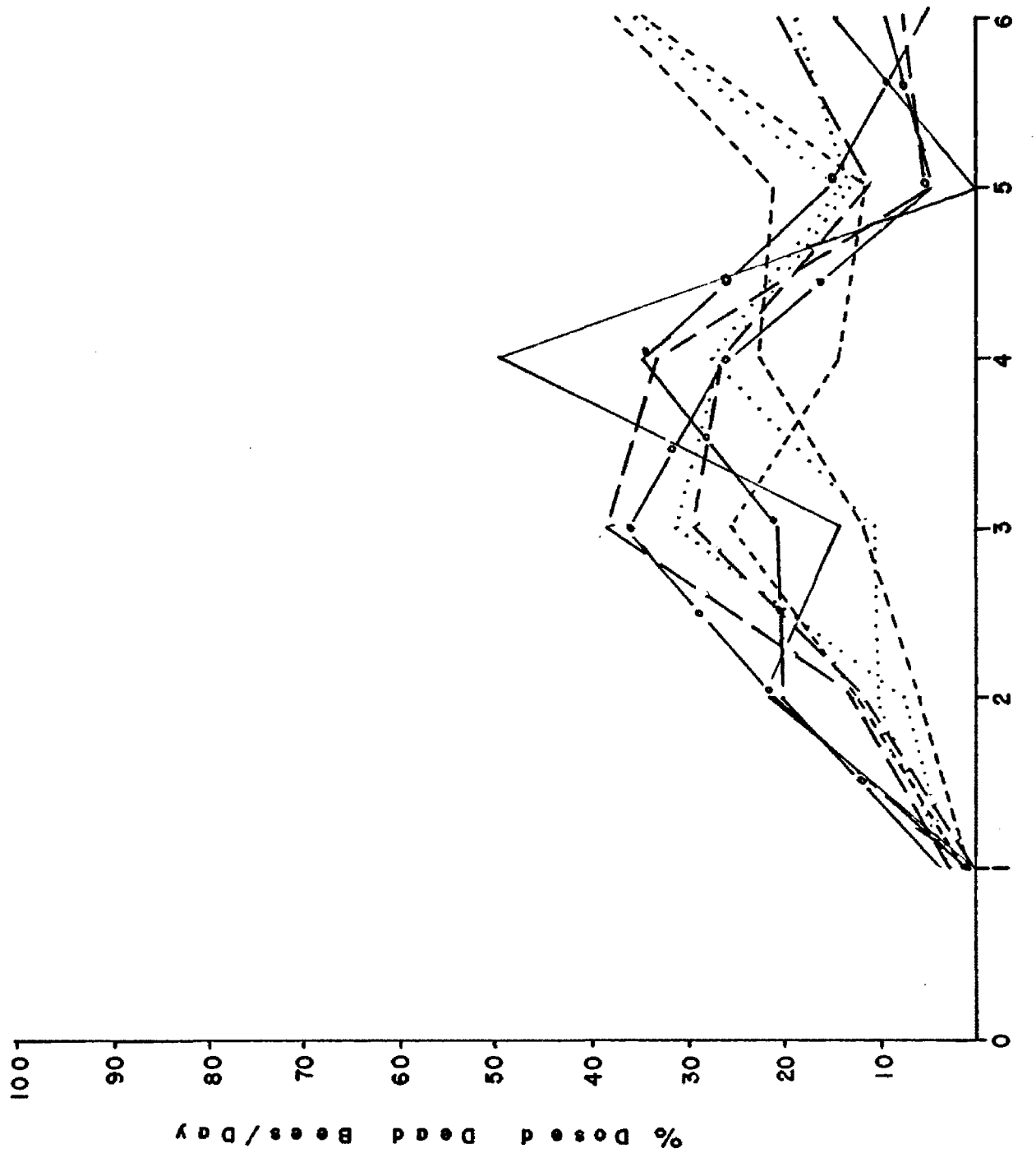


Fig. 5. Test 4 - As_2O_3. Theoretical Dose: 24 hr - 3.0 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 2.28 ug/bee.

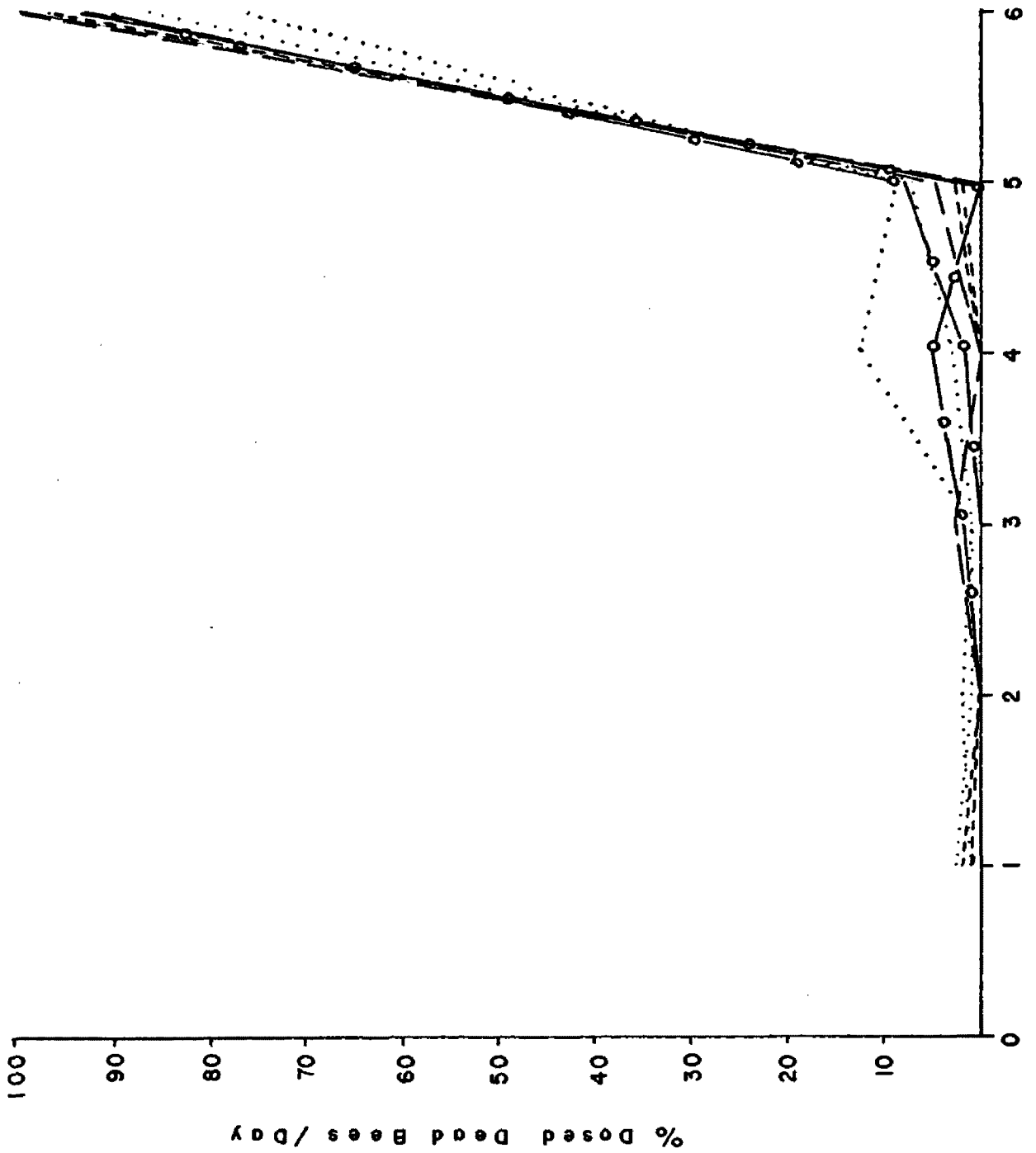


Fig. 6. Test 5 - As_2O_3. Theoretical Dose: 24 hr - 0.42 ug/bee. Actual Dose (Mean Calcconc): 24 hr - 0.33 ug/bee.

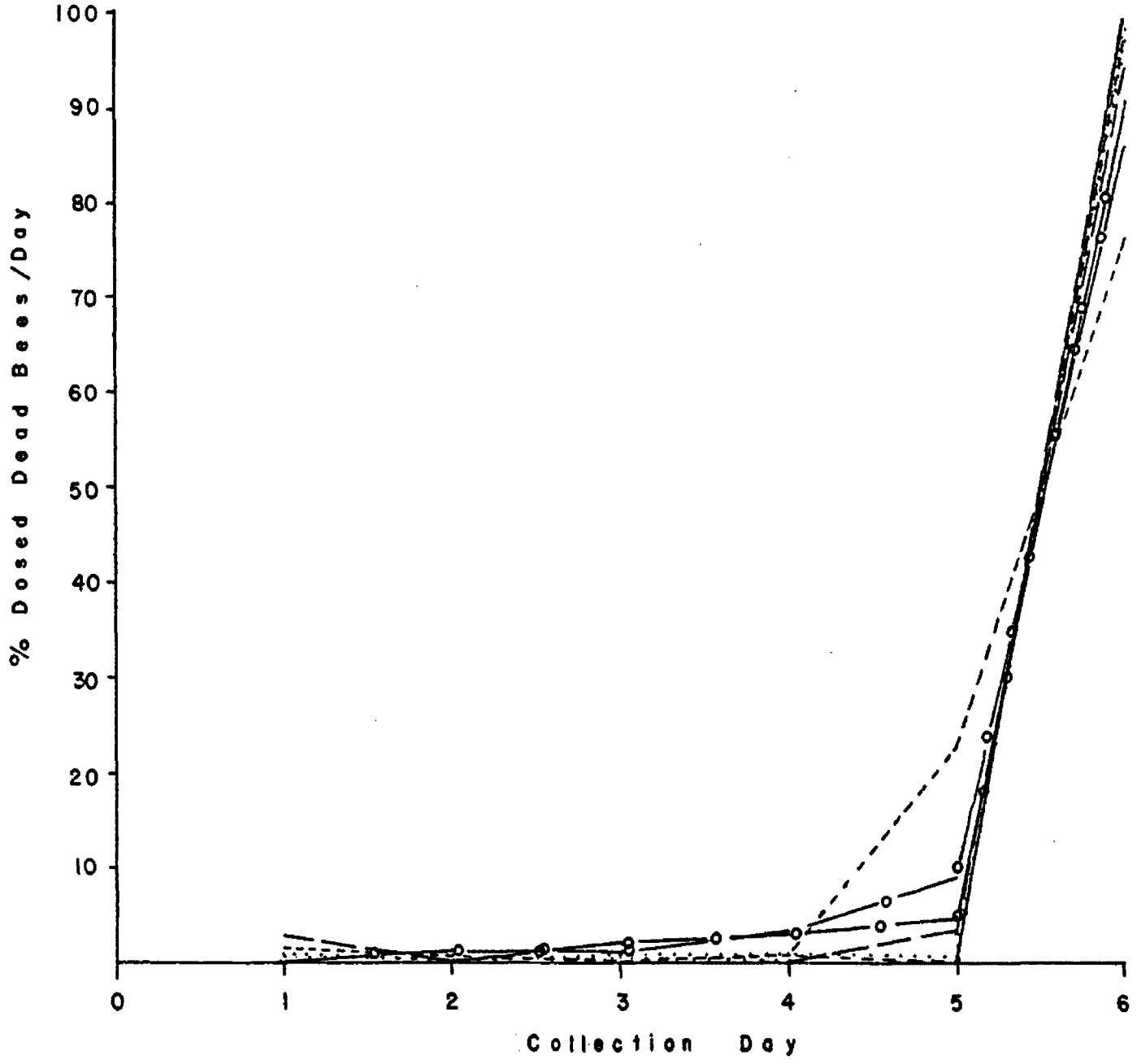


Fig. 7. Test 6 - As_2O_3. Theoretical Dose: 24 hr - 0.06 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.04 ug/bee.

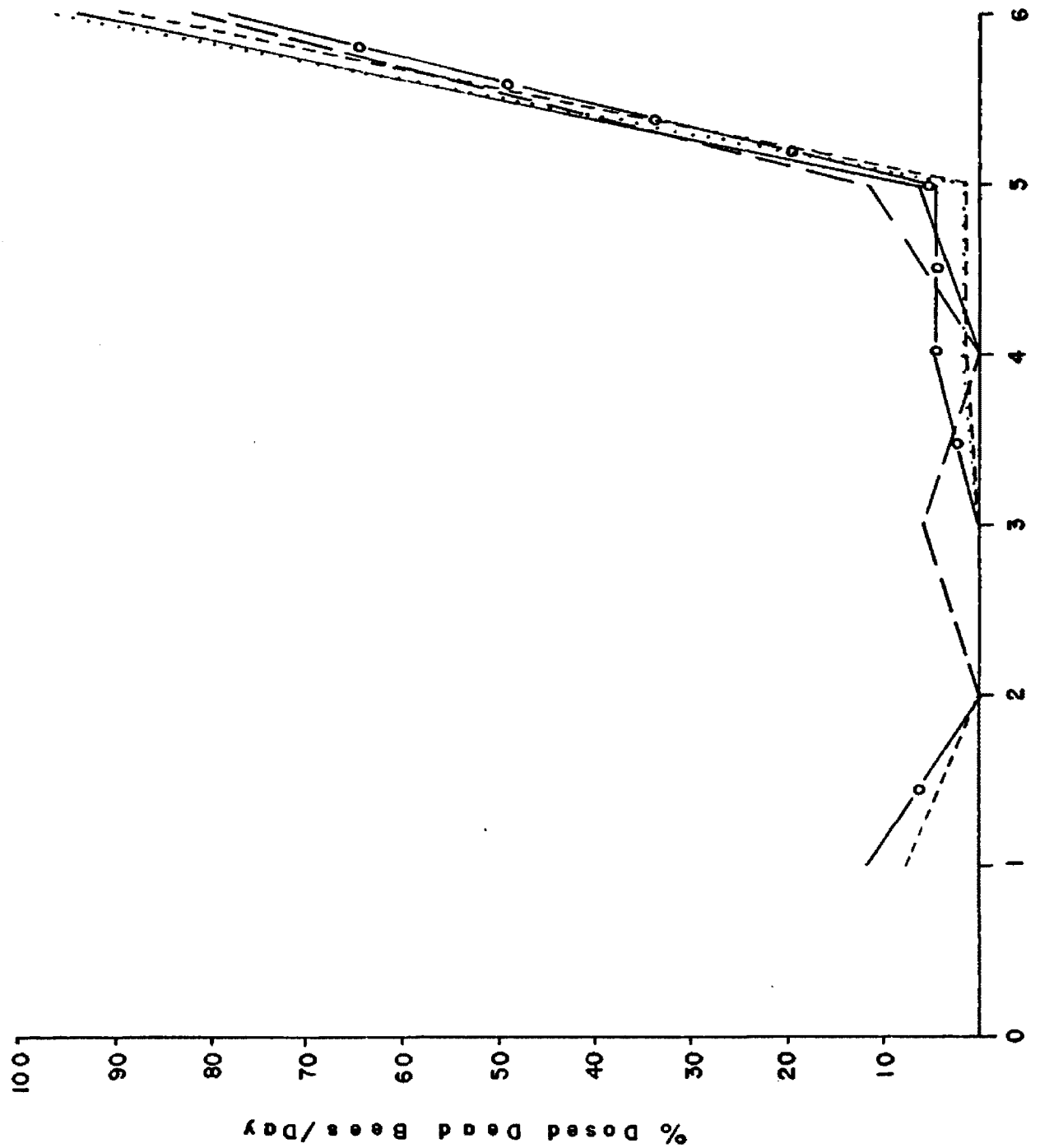


Fig. 8 Control: Tests 4-6. Theoretical Dose: 0.0 ug/bee.

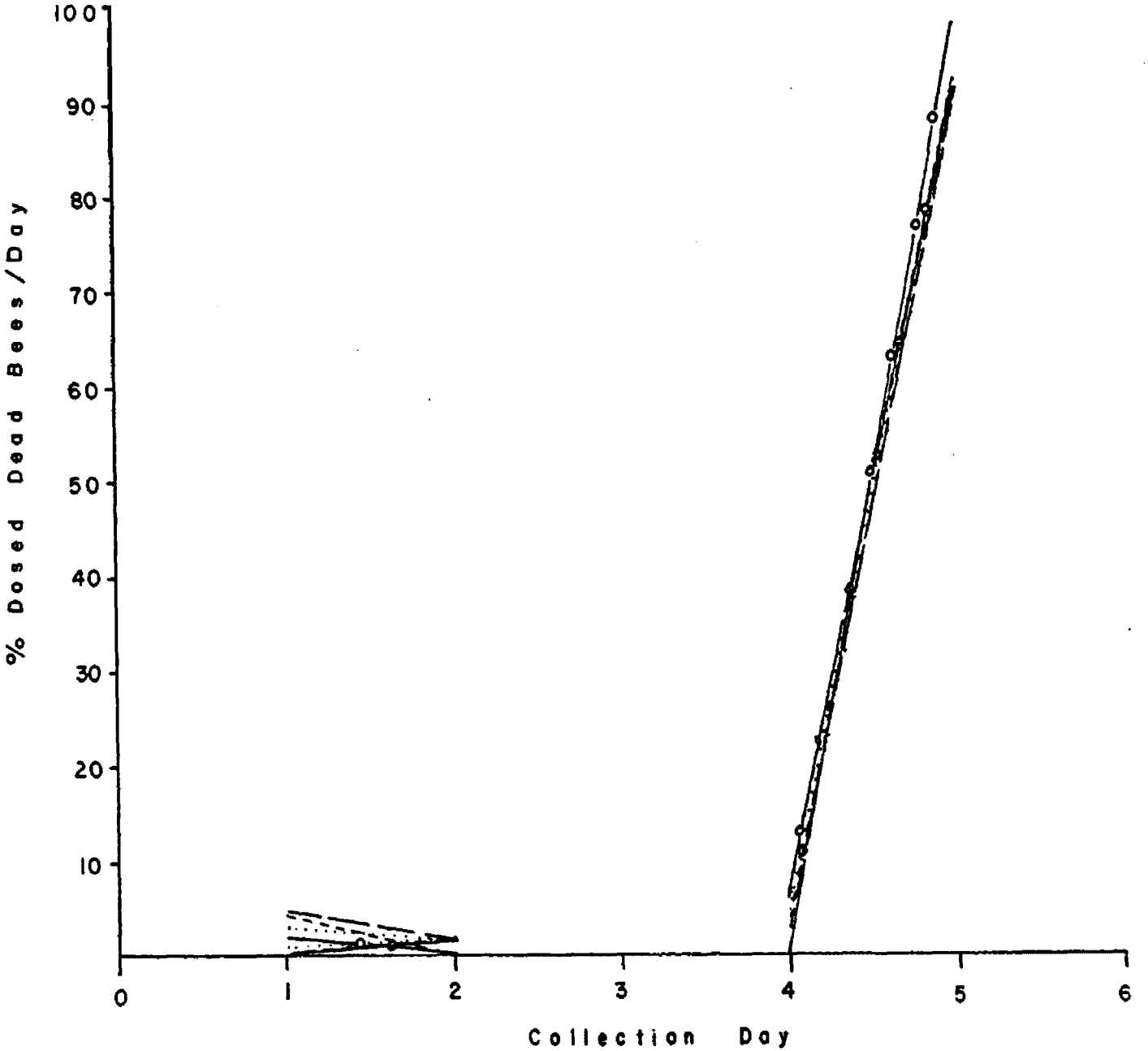


Fig. 9. Test 7 - $NaAsO_2$. Theoretical Dose: 24 hr - 0.50 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.05 ug/bee.

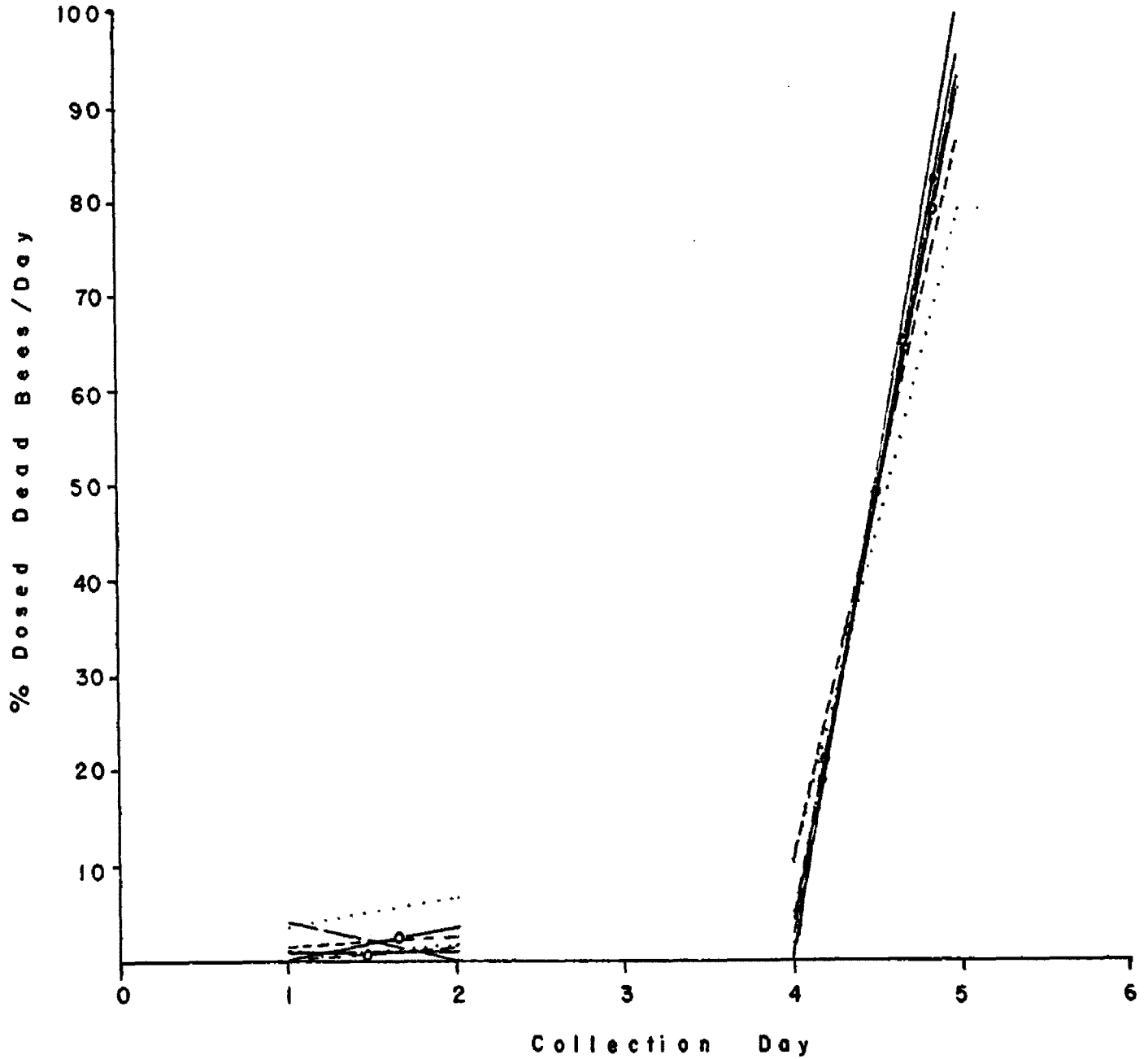


Fig. 10. Test 8 - $NaAsO_2$. Theoretical Dose: 24 hr - 0.07 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.006 ug/bee.

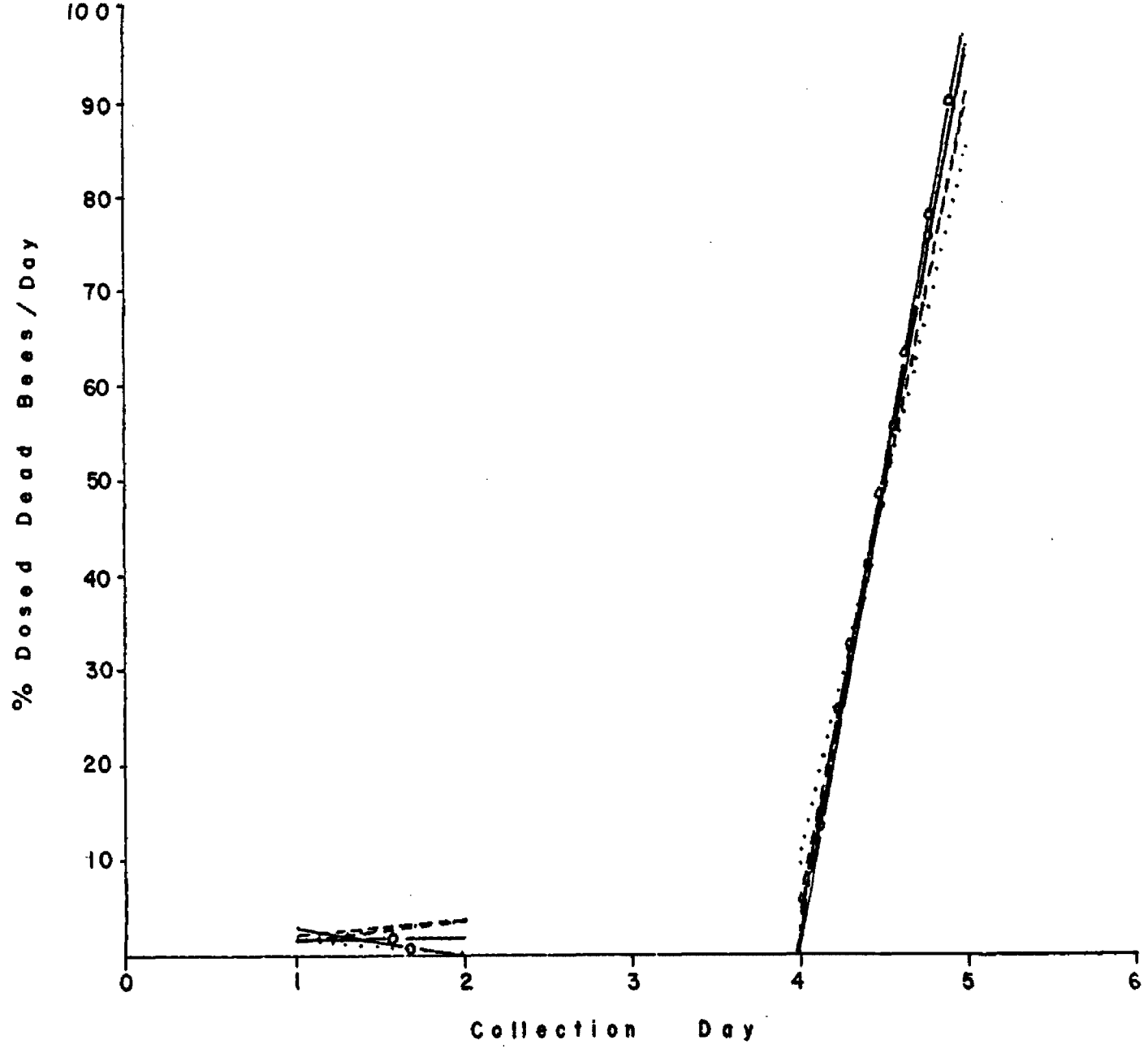


Fig. 11. Test 9 - $NaAsO_2$. Theoretical Dose: 24 hr - 0.01 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.001 ug/bee.

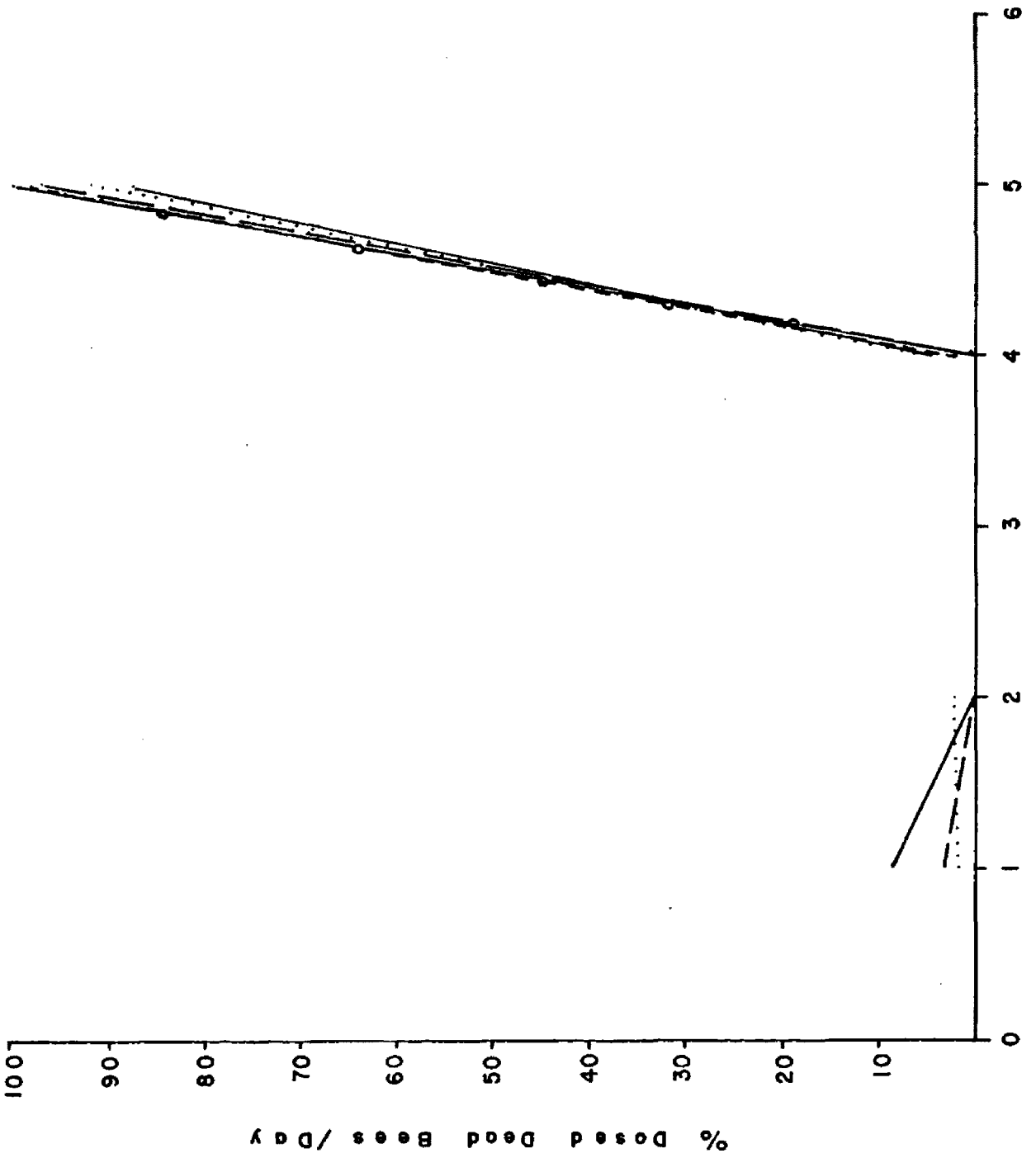


Fig. 12. Control: Tests 7-9. Theoretical Dose: 0.0 ug/bee.

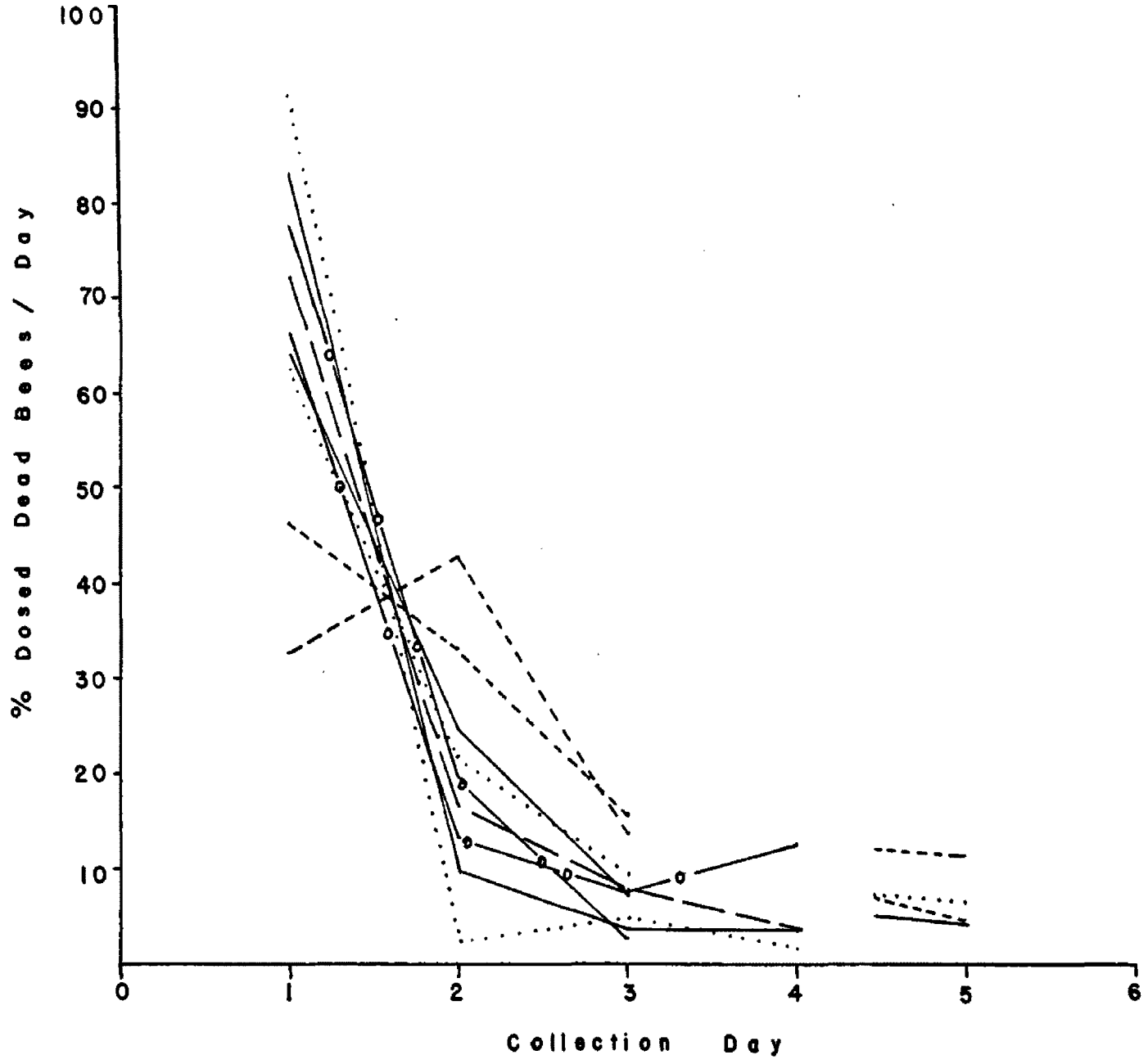


Fig. 13. Test 10 - $NaAsO_2$. Theoretical Dose: 24 hr - 10.0 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.65 ug/bee.

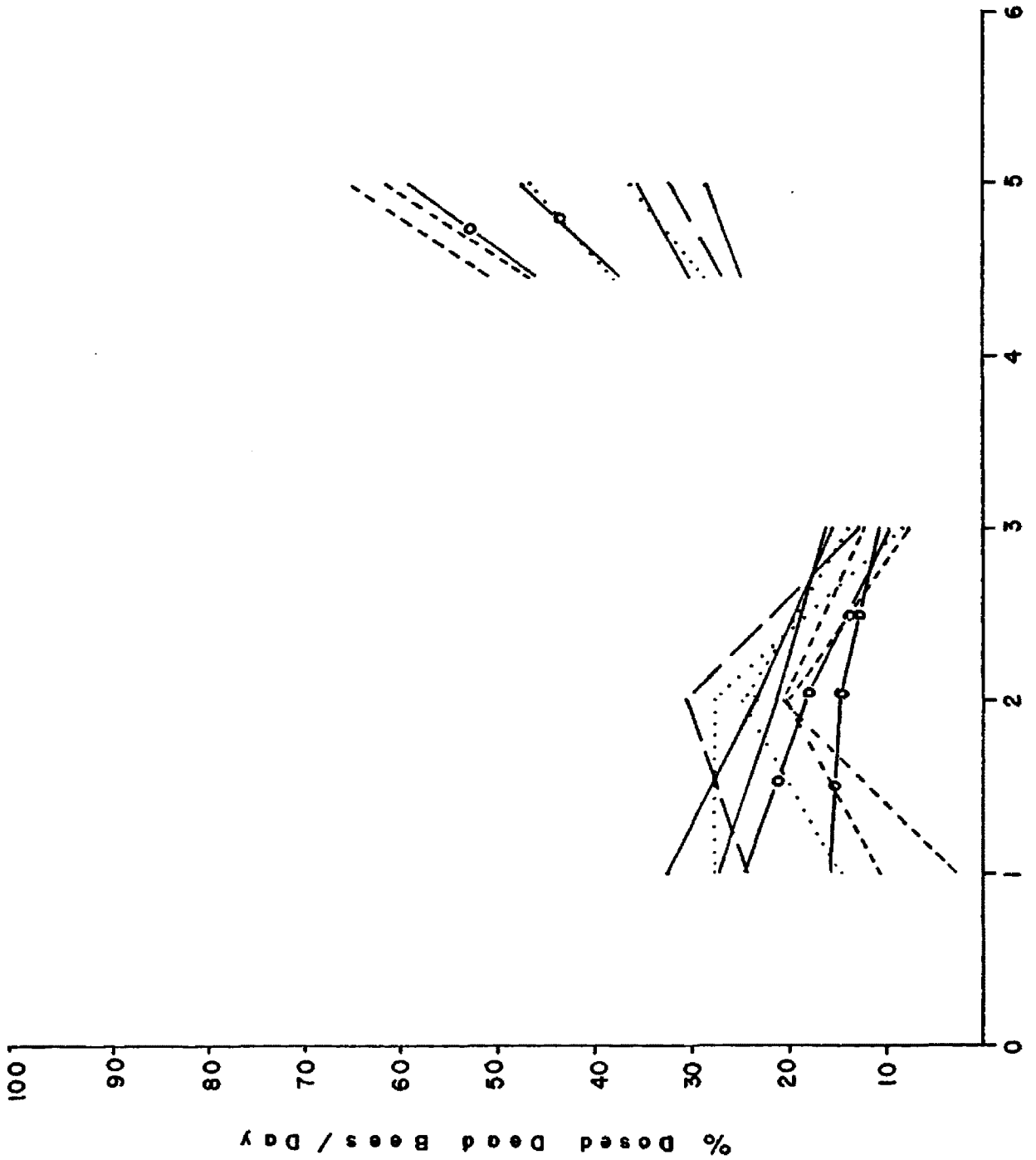


Fig. 14. Test 11 - $NaAsO_2$. Theoretical Dose: 24 hr - 5.0 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.46 ug/bee.

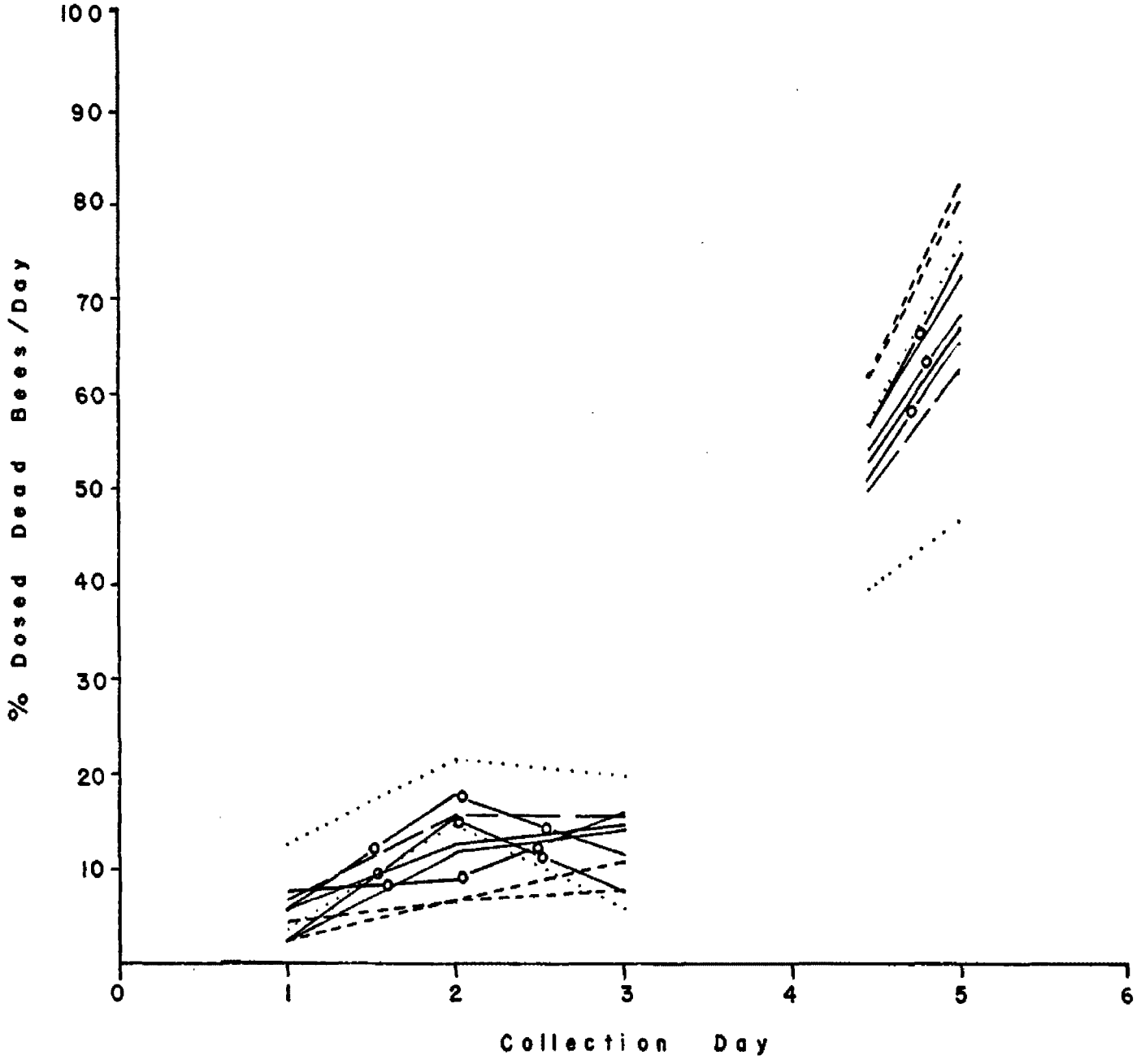


Fig. 15. Test 12 - $NaAsO_2$. Theoretical Dose: 24 hr - 3.0 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.35 ug/bee.

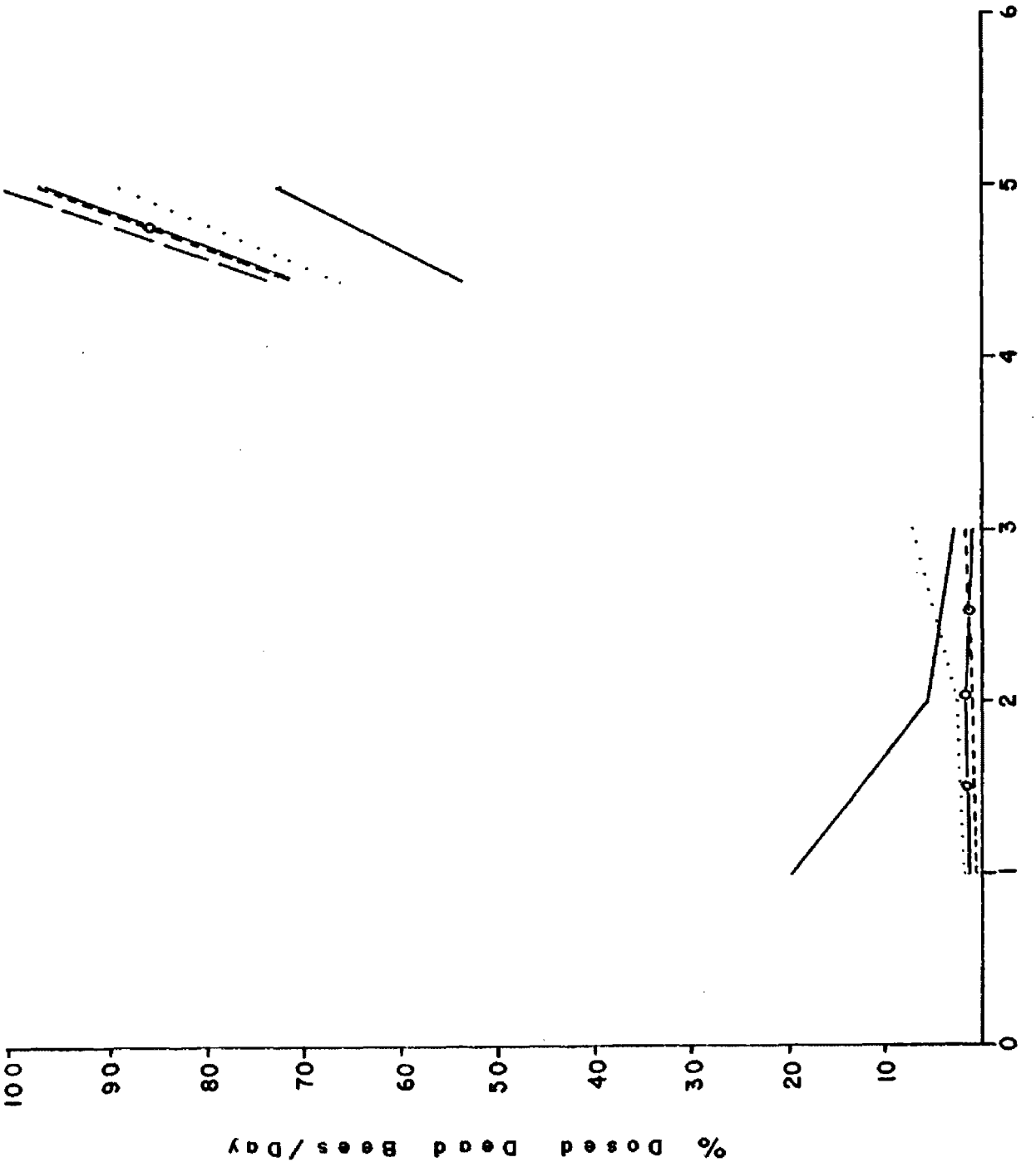


Fig. 16. Control: Tests 10-12. Theoretical Dose: 0.0 ug/bee.

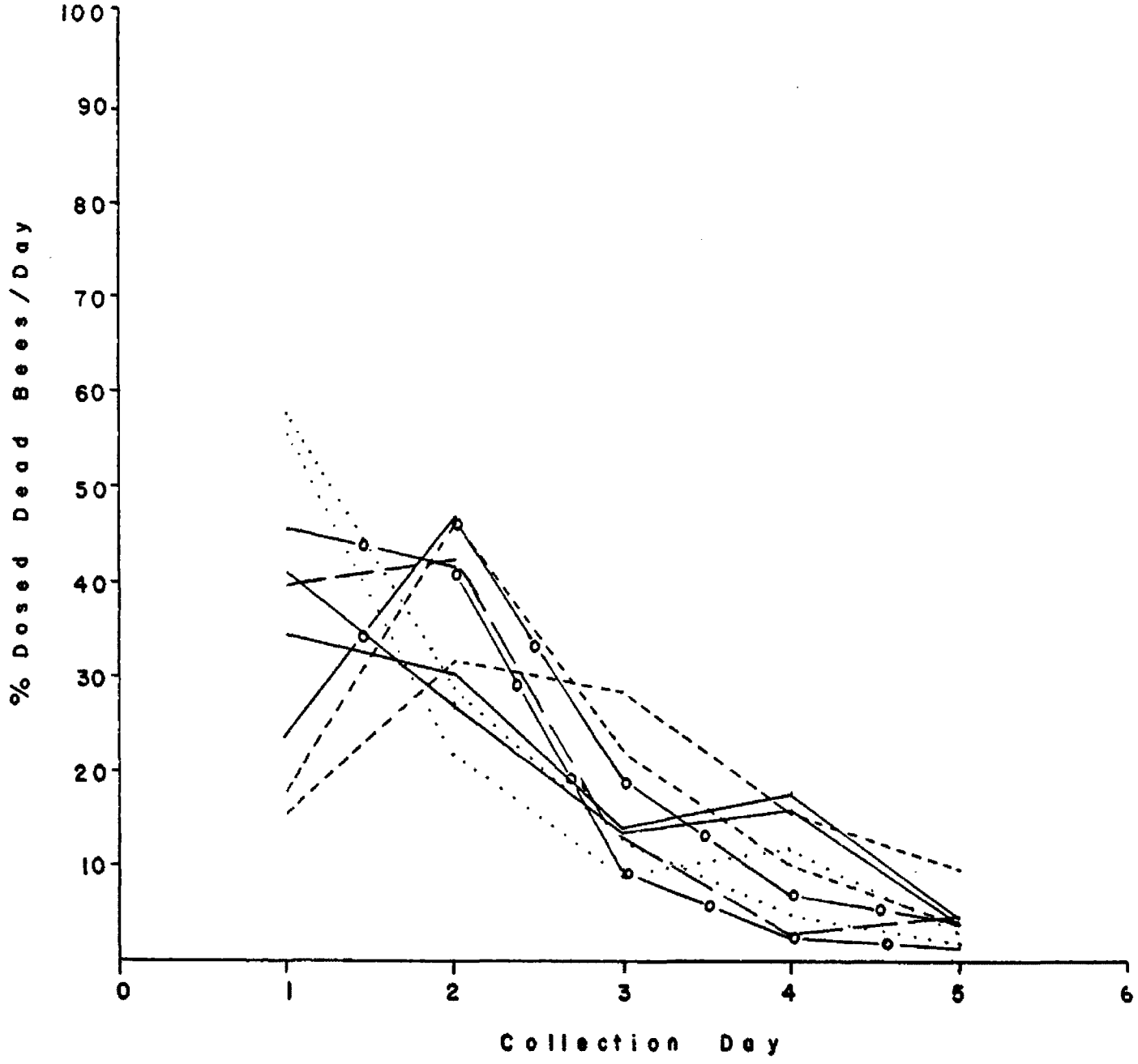


Fig. 17. Test 13 - $NaAsO_2$. Theoretical Dose: 24 hr - 8.0 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.49 ug/bee.

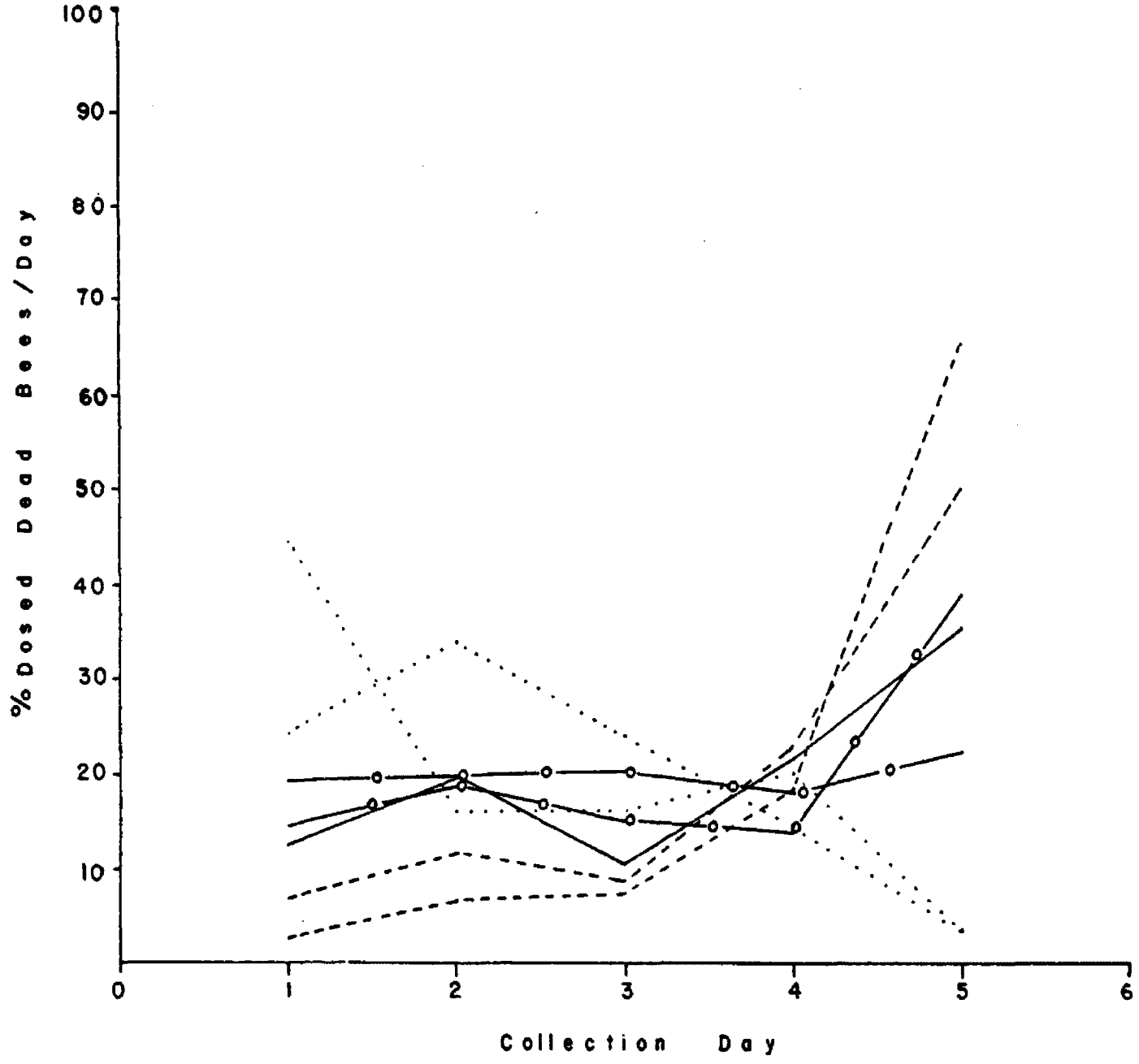


Fig. 18. Test 14 - $NaAsO_2$. Theoretical Dose: 24 hr - 7.0 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.47 ug/bee.

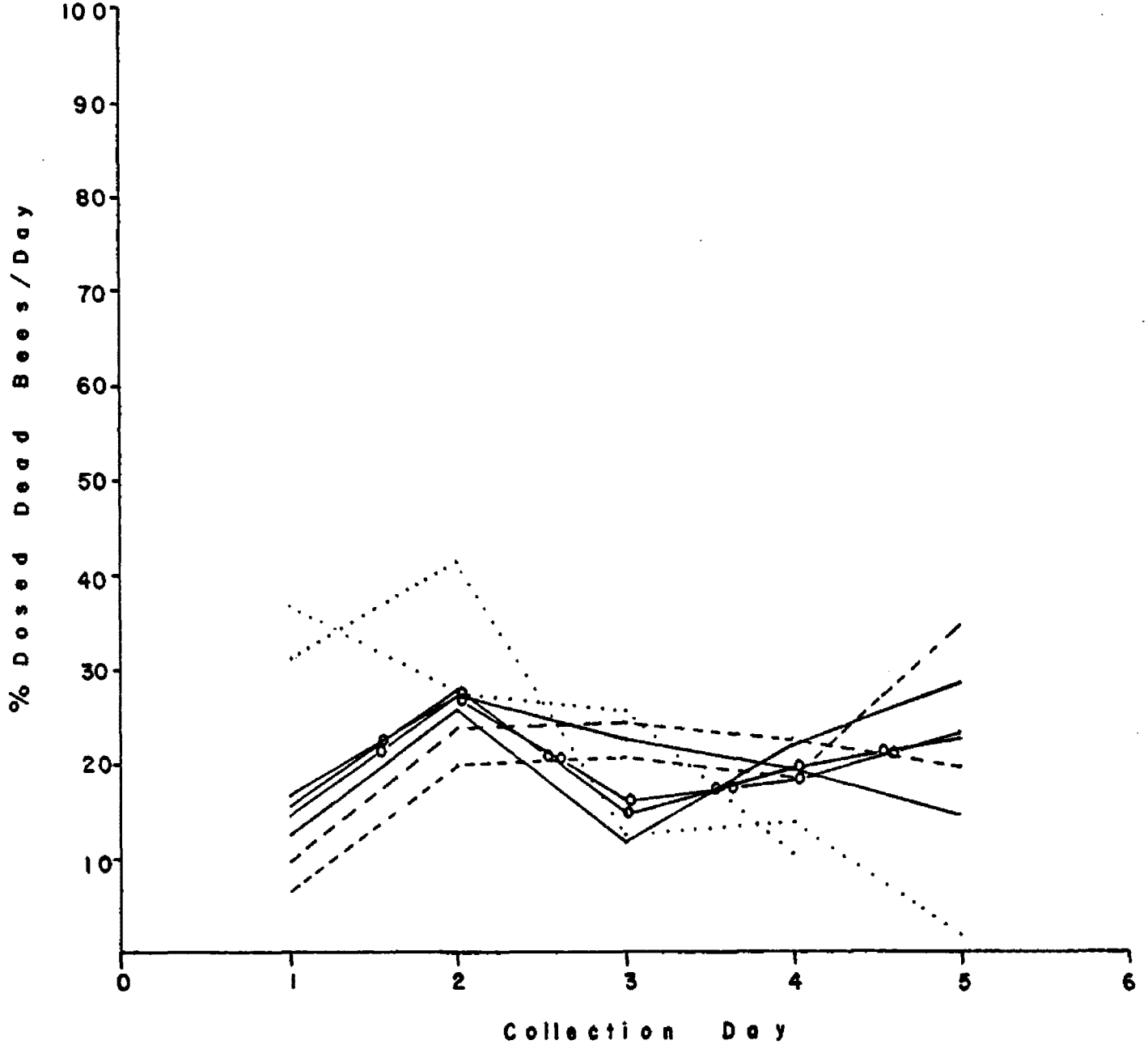


Fig. 19. Test 15 - $NaAsO_2$. Theoretical Dose: 24 hr - 6.0 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.46 ug/bee.

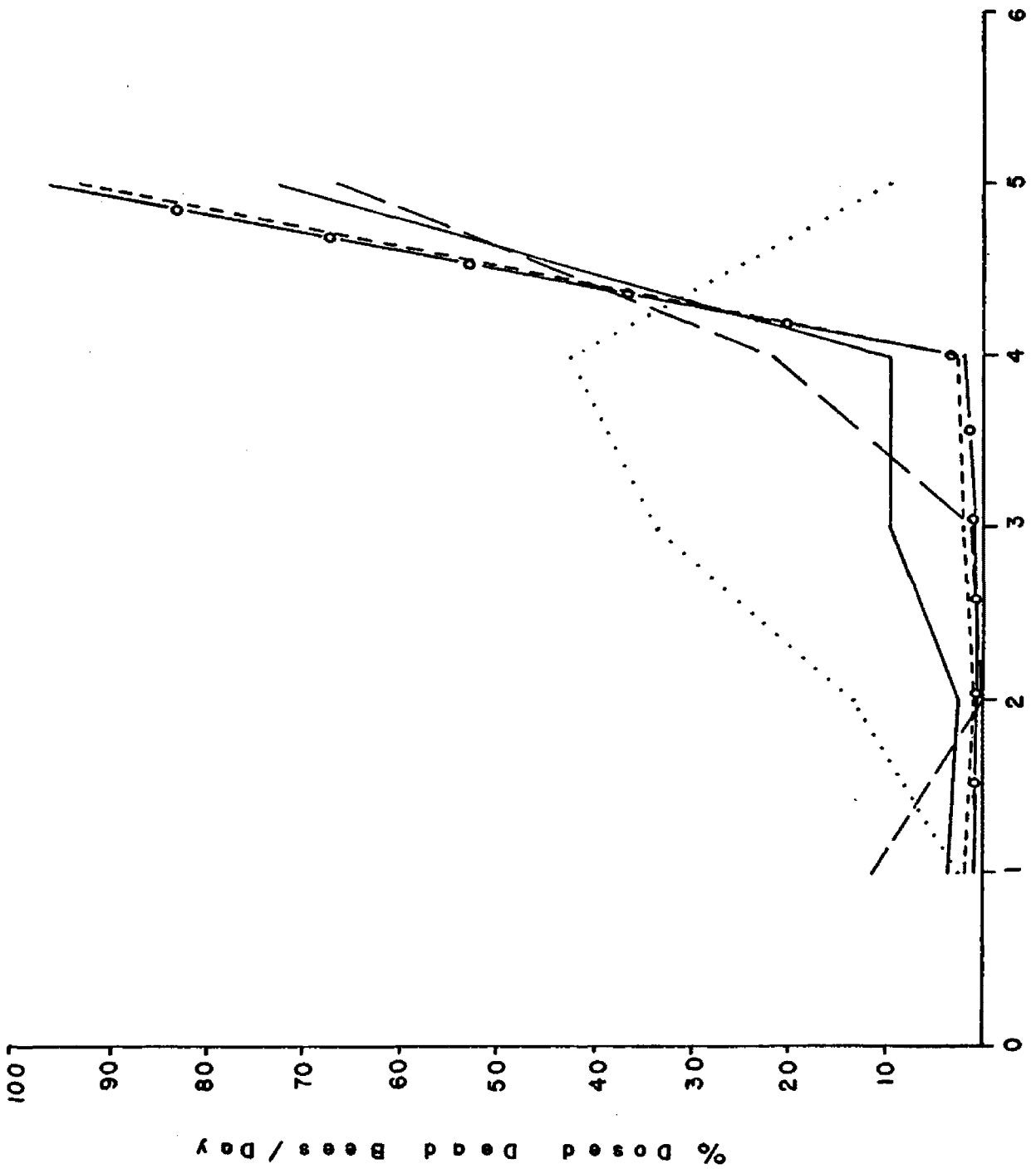


Fig. 20. Control: Tests 13-15. Theoretical Dose: 0.0 ug/bee.

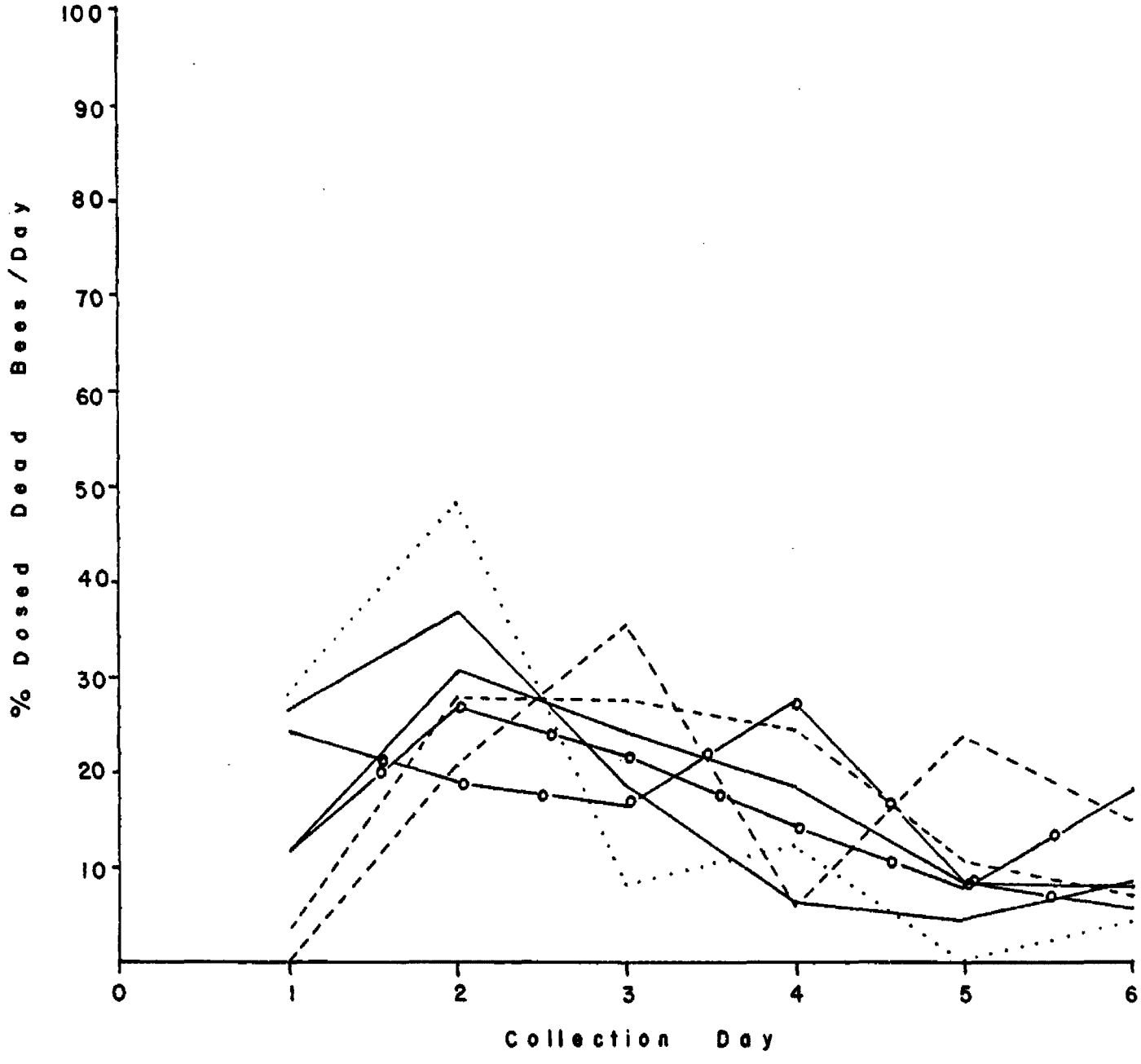


Fig. 21. Test 16 - $NaAsO_2$. Theoretical Dose: 24 hr - 4.0 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.34 ug/bee.

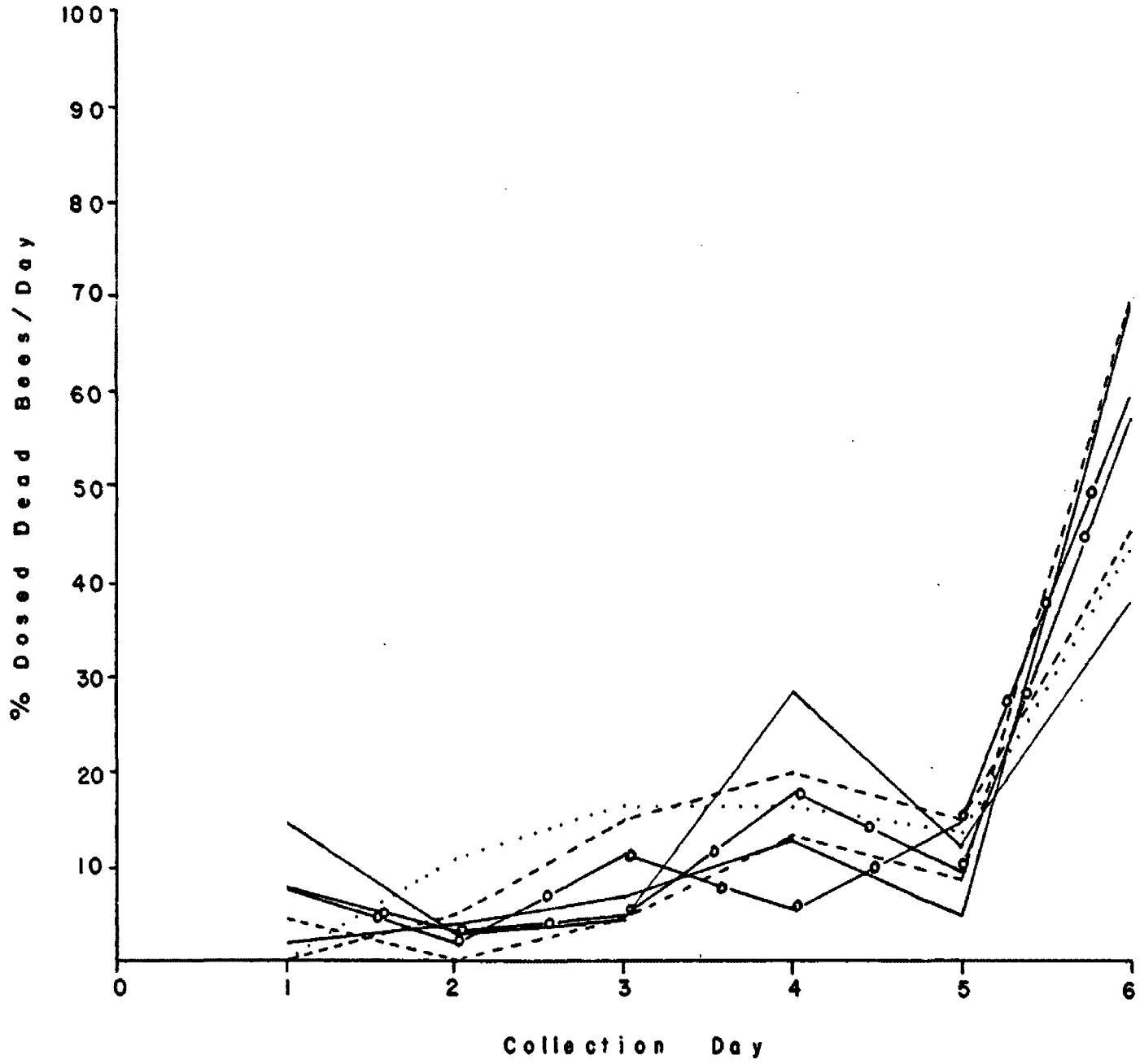


Fig. 22. Test 17 - $NaAsO_2$. Theoretical Dose: 24 hr - 2.0 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.19 ug/bee.

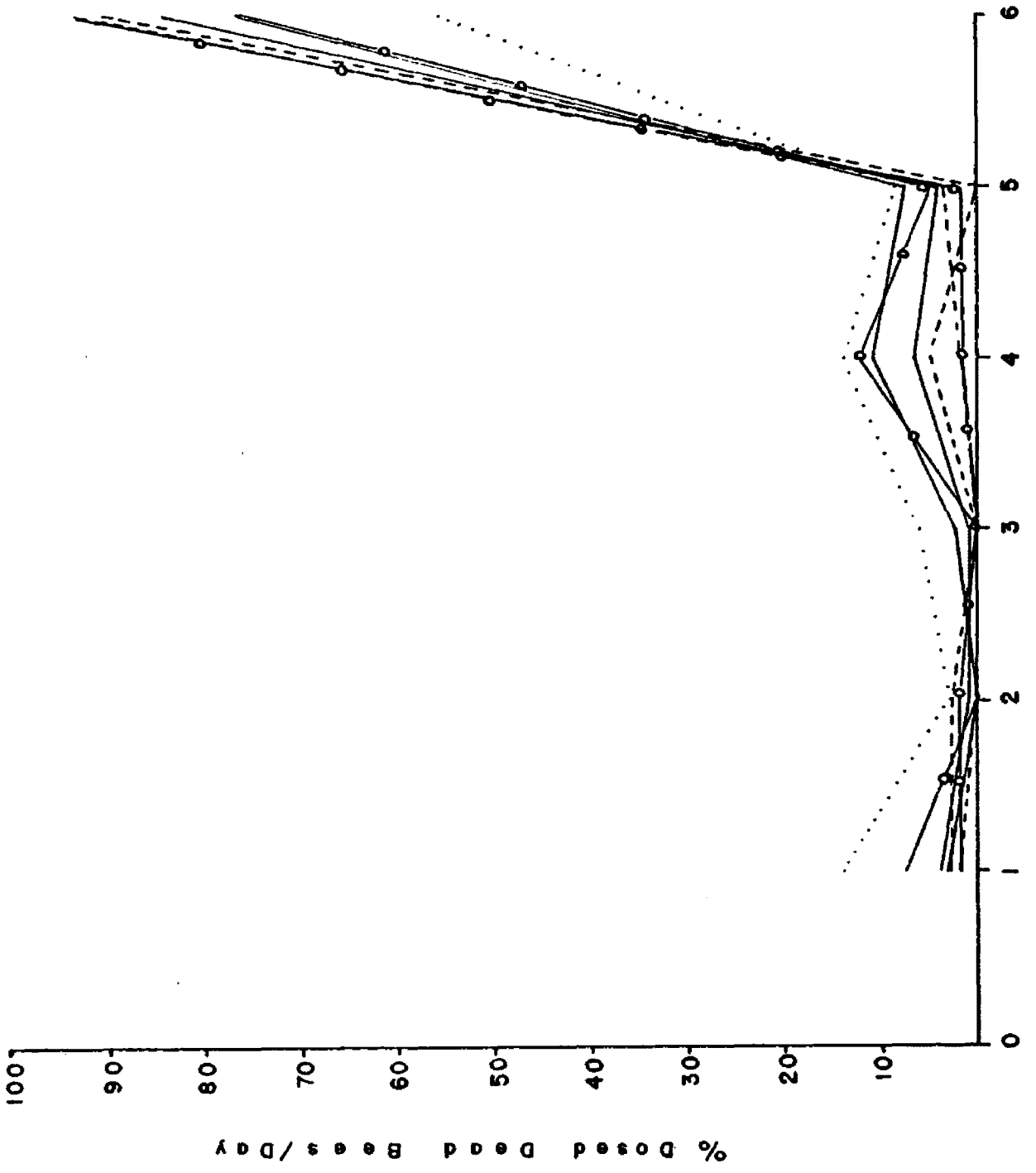


Fig. 23. Test 18 - $NaAsO_2$. Theoretical Dose: 24 hr - 1.0 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.12 ug/bee.

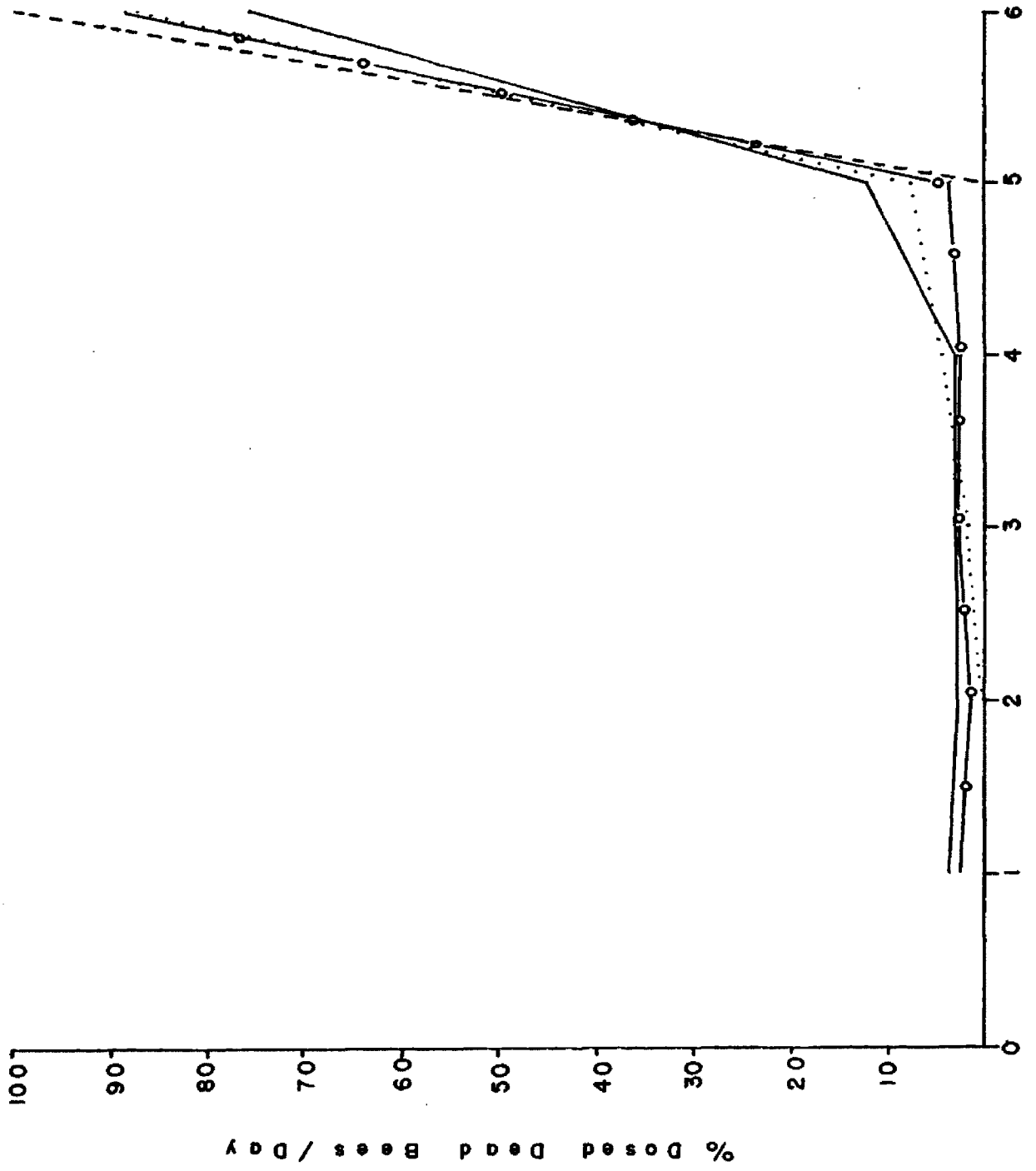


Fig. 24. Control: Tests 16-18. Theoretical Dose : 0.0 ug/bee.

APPENDIX B

ACCUMULATIVE % DOSED DEAD BEES/DAY MORTALITY RESPONSE GRAPHS

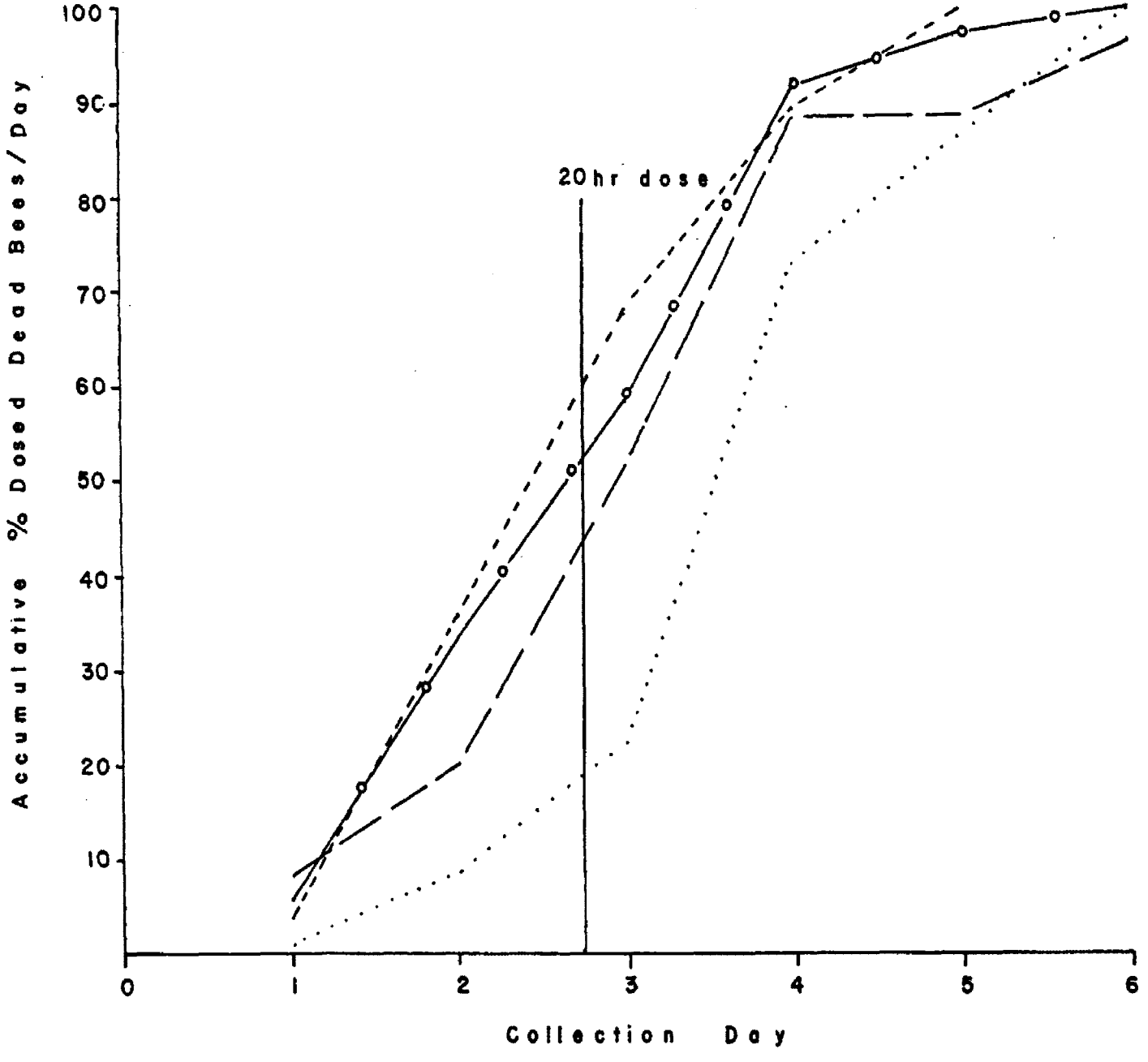


Fig. 1. Test 1 - As_2O_3. Theoretical Dose: 4 hr - 0.5 ug/bee, 24 hr - 3.0 ug/bee. Actual Dose (Mean Calcconc): 24 hr - 0.22 ug/bee.

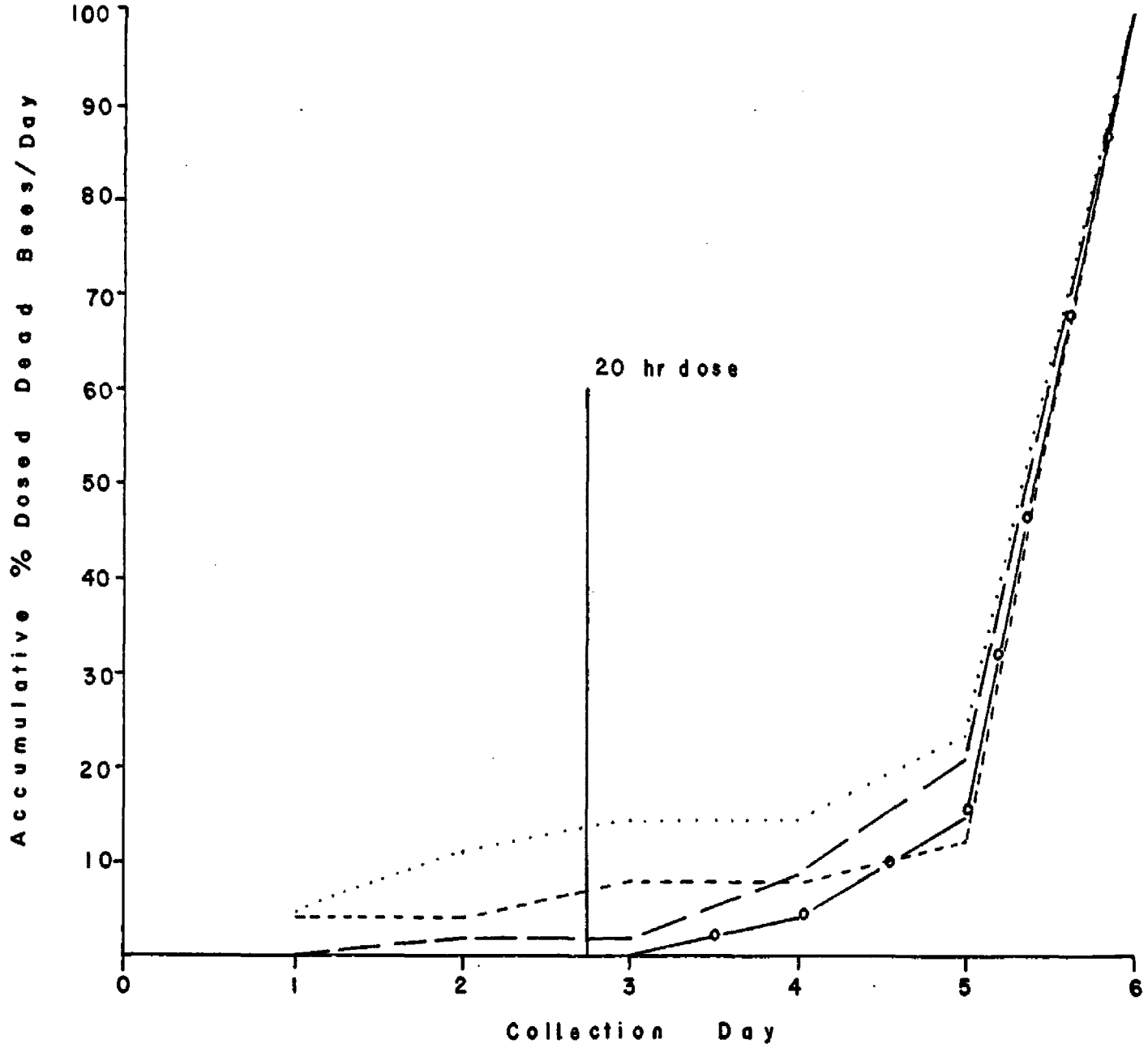


Fig. 2. Test 2 - As_2O_3. Theoretical Dose: 4 hr - 0.07 ug/bee, 24 hr - 0.42 ug/bee. Actual Dose (Mean Calcconc): 24 hr - 0.087 ug/bee.

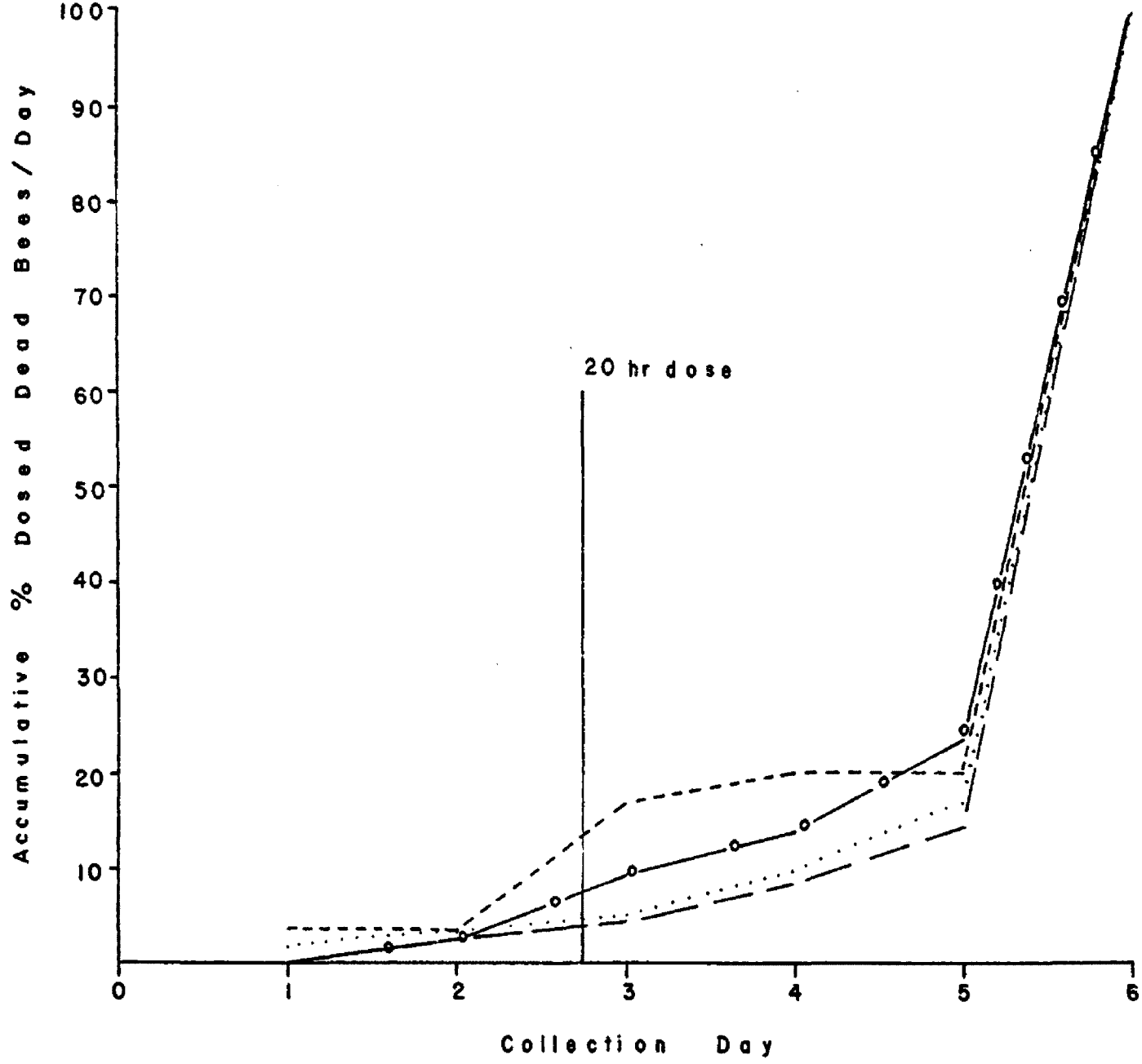


Fig. 3 Test 3 - As_2O_3. Theoretical Dose: 4 hr - 0.01 ug/bee,24 hr - 0.06 ug/bee. Actual Dose (Mean Calcconc): 24 hr - 0.09 ug/bee.

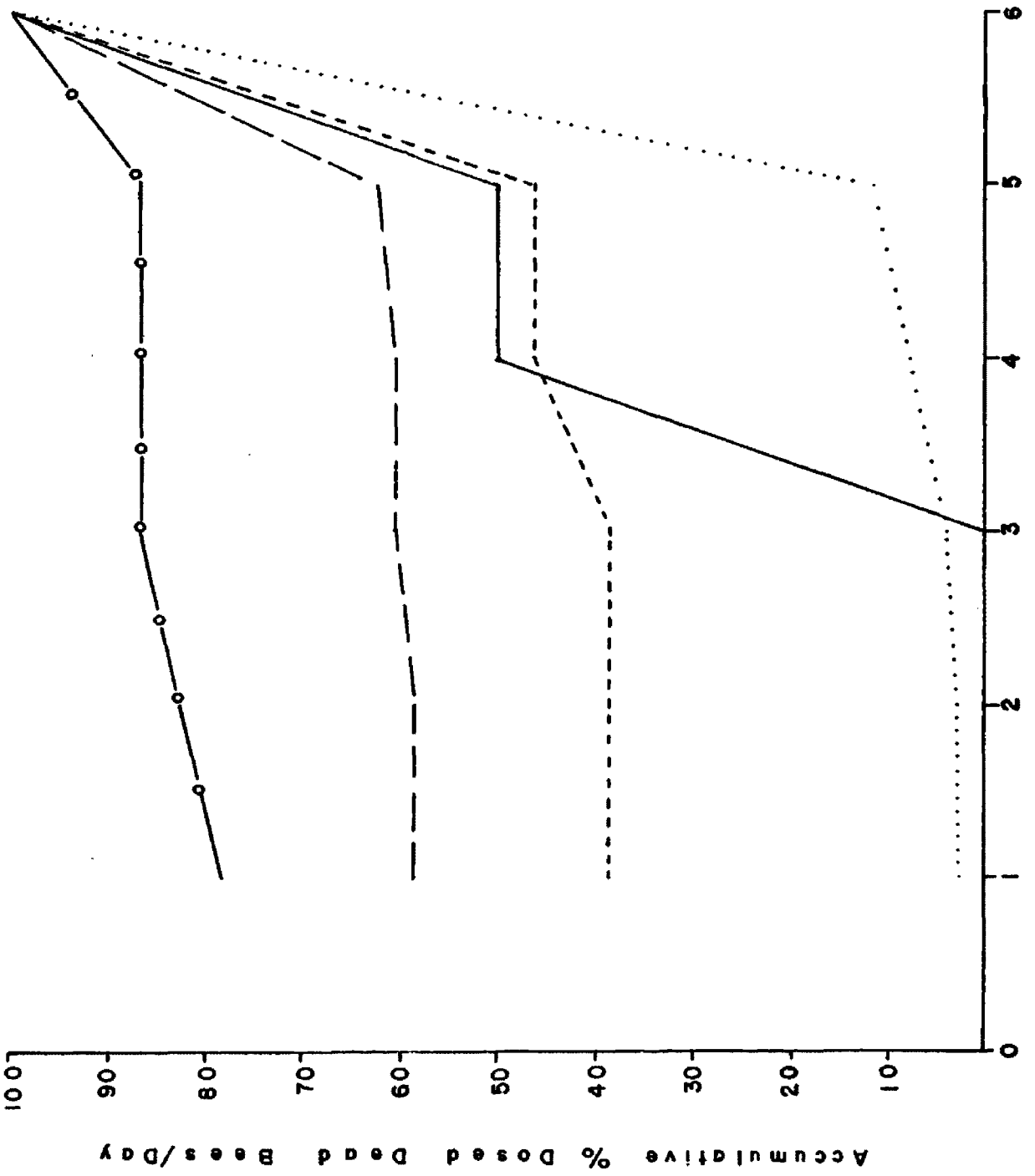


Fig. 4. Control: Tests 1-3. Theoretical Dose: 0.0 ug/bee.

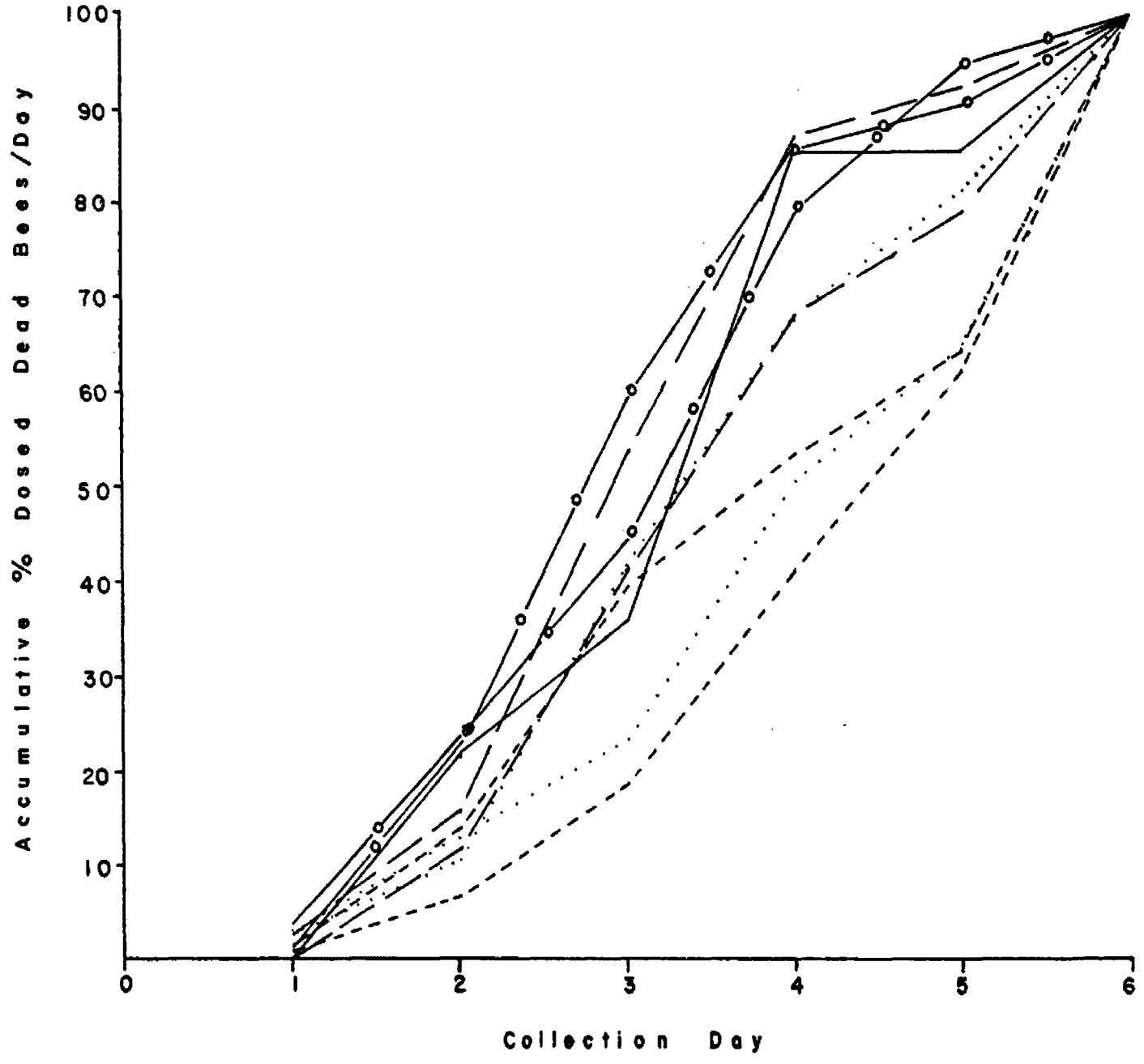


Fig. 5 Test 4 - As_2O_3. Theoretical Dose: 24 hr - 3.0 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 2.28 ug/bee.

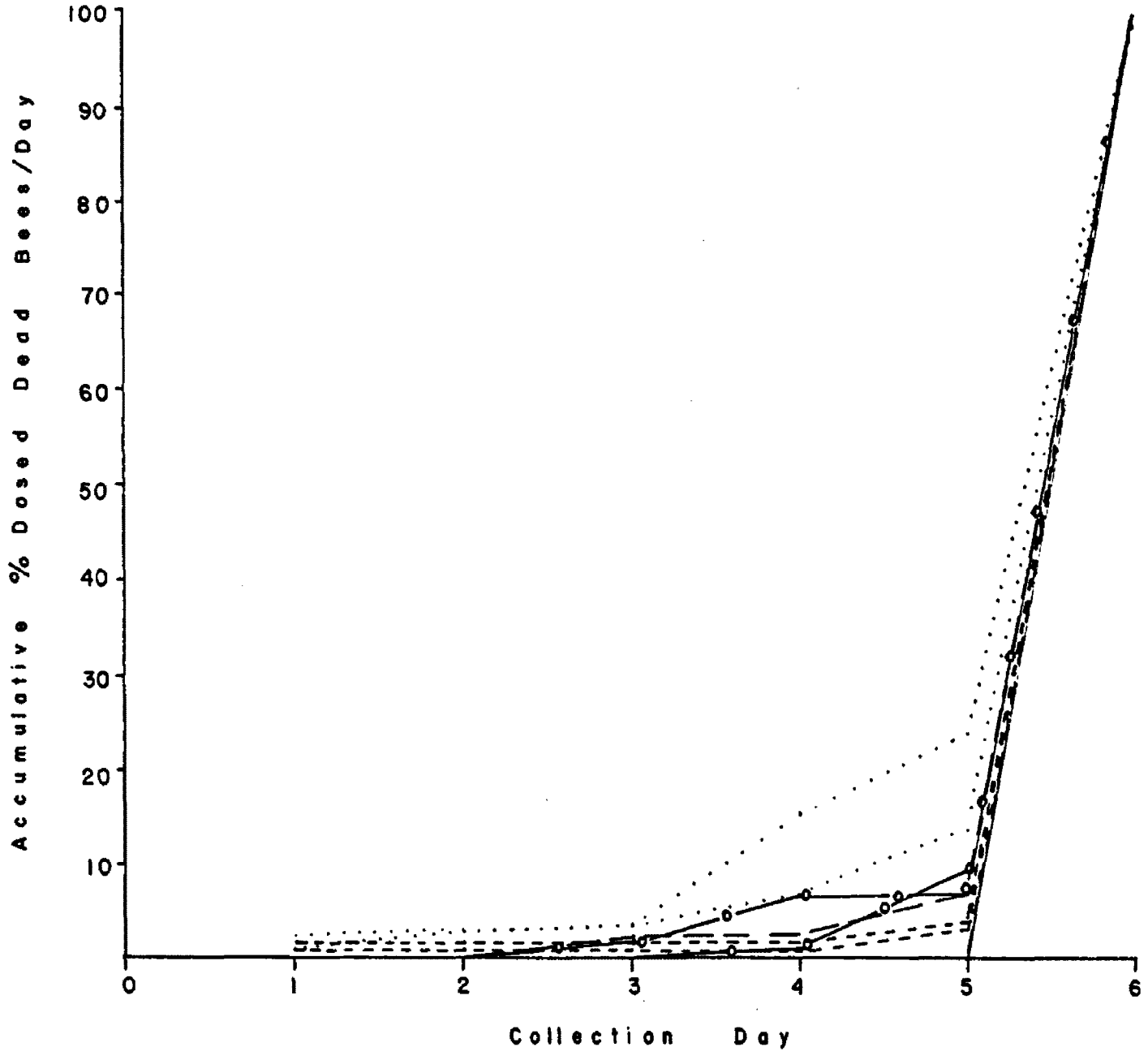


Fig. 6. Test 5 - As_2O_3. Theoretical Dose: 24 hr - 0.42 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.33 ug/bee.

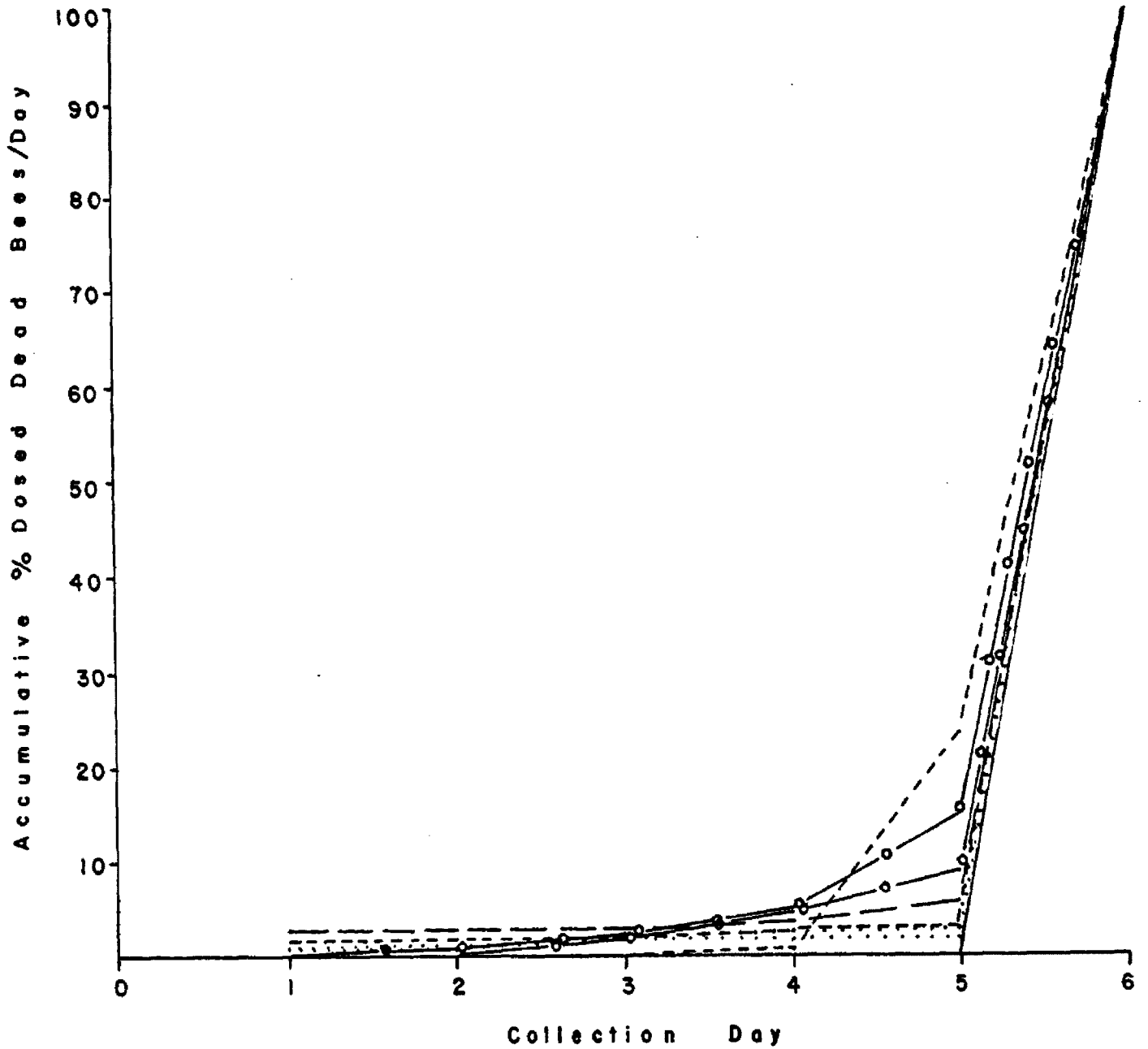


Fig. 7. Test 6 - As_2O_3. Theoretical Dose: 24 hr - 0.06 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.04 ug/bee.

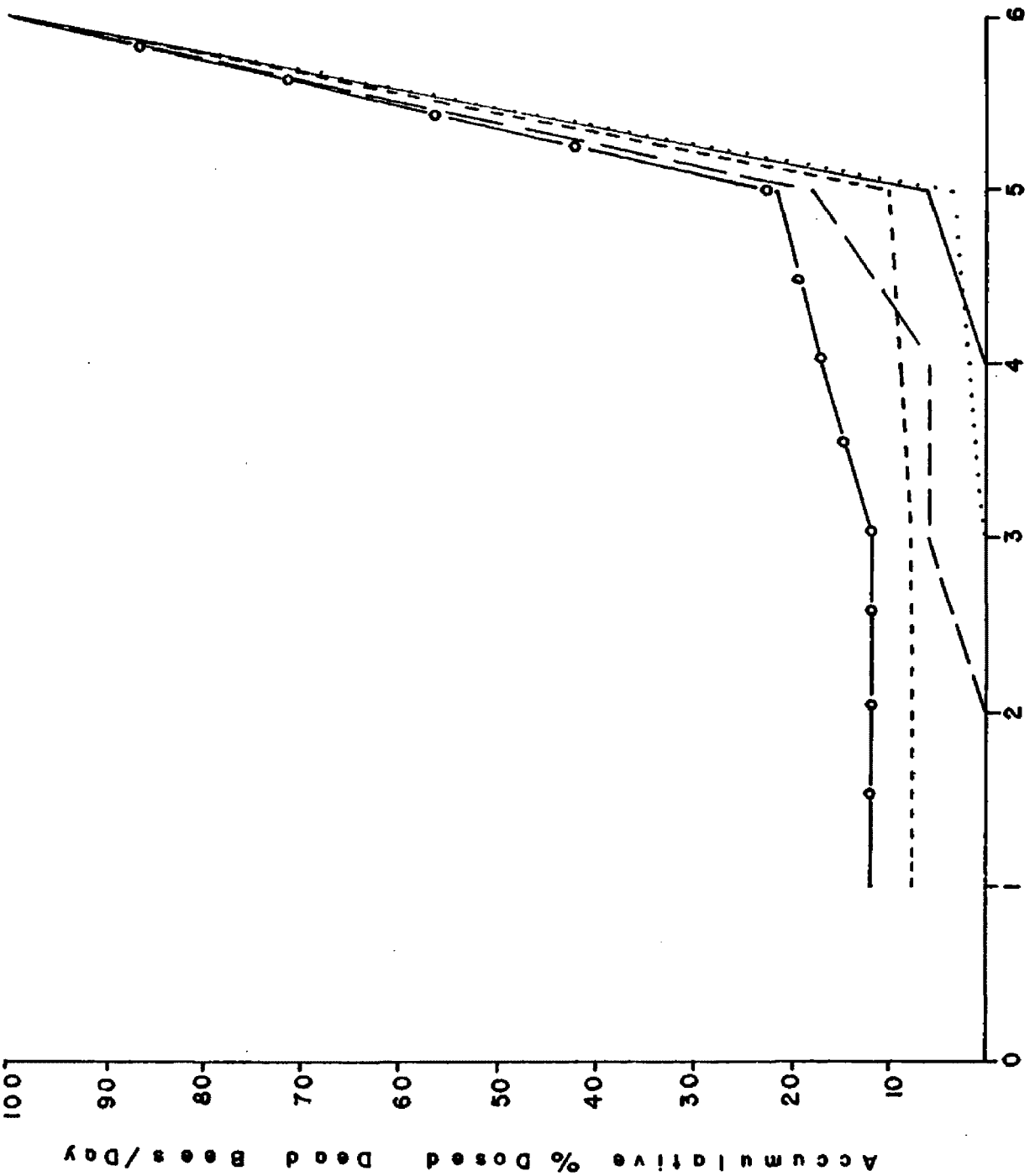


Fig. 8. Control: Tests 4-6. Theoretical Dose: 0.0 ug/bee.

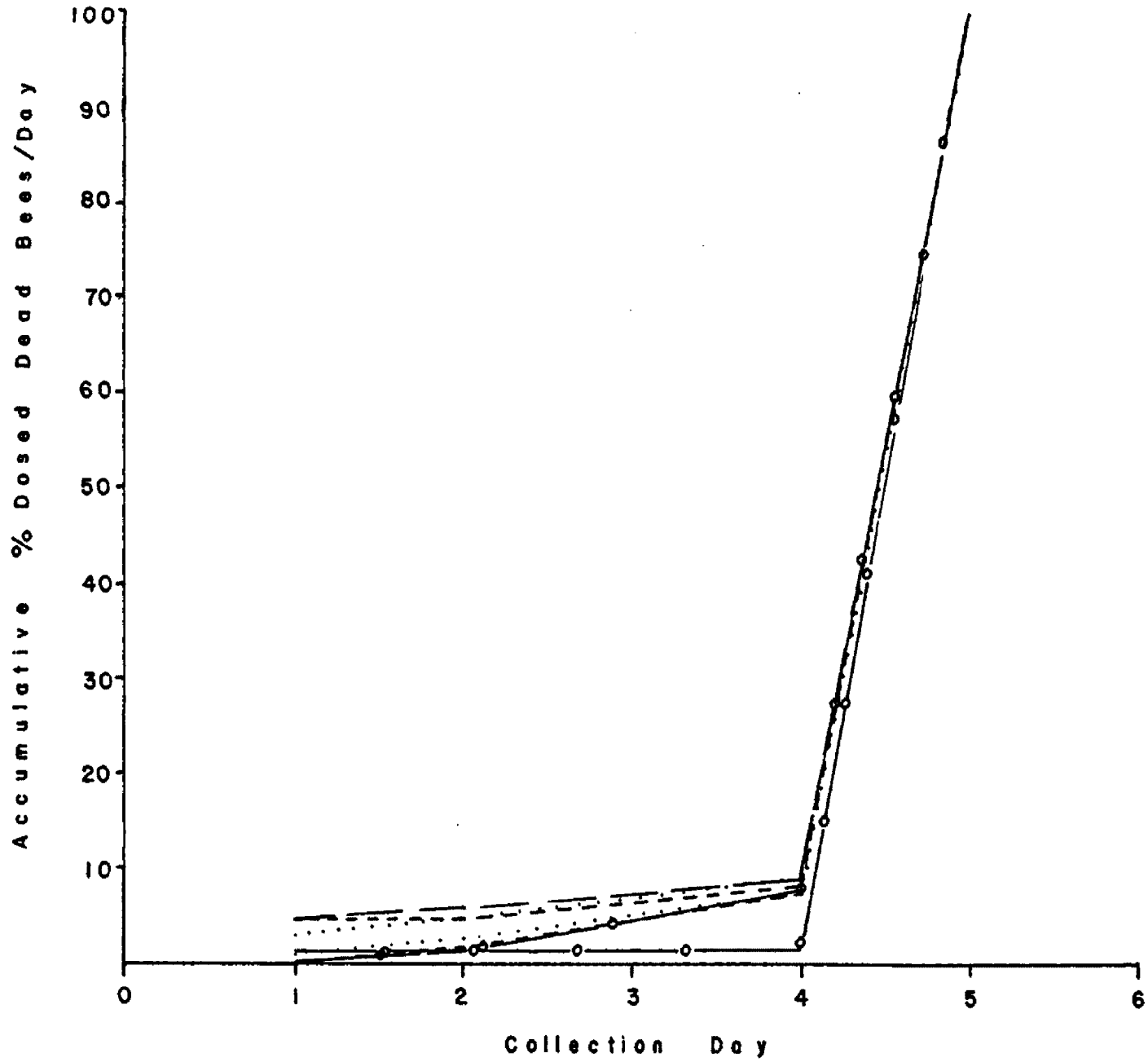


Fig. 9. Test 7 - $NaAsO_2$. Theoretical Dose: 24 hr - 0.5 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.05 ug/bee.

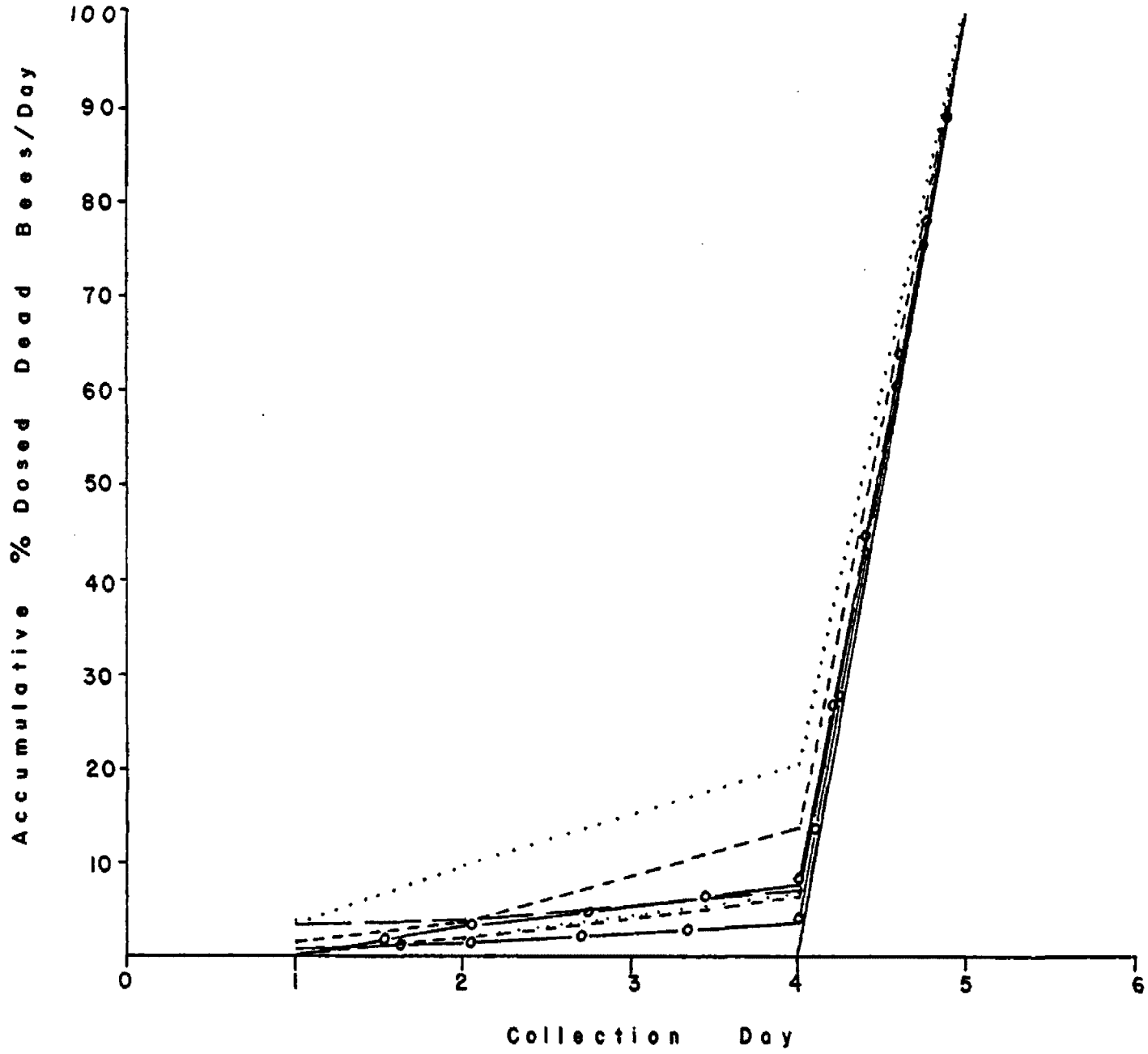


Fig. 10. Test 8 - $NaAsO_2$. Theoretical Dose: 24 hr - 0.07 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.006 ug/bee.

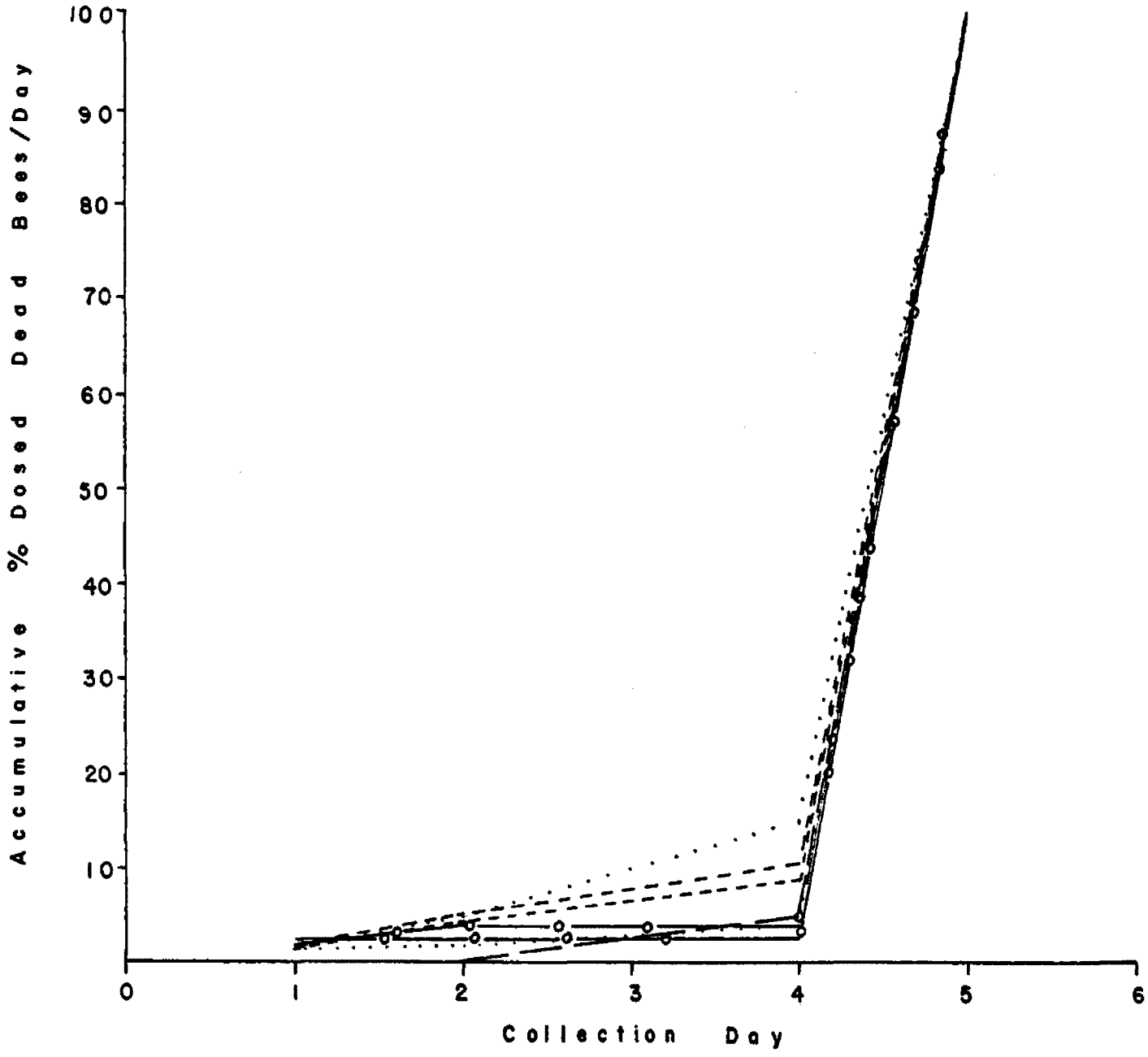


Fig. 11. Test 9 - $NaAsO_2$. Theoretical Dose: 24 hr - 0.01 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.001 ug/bee.

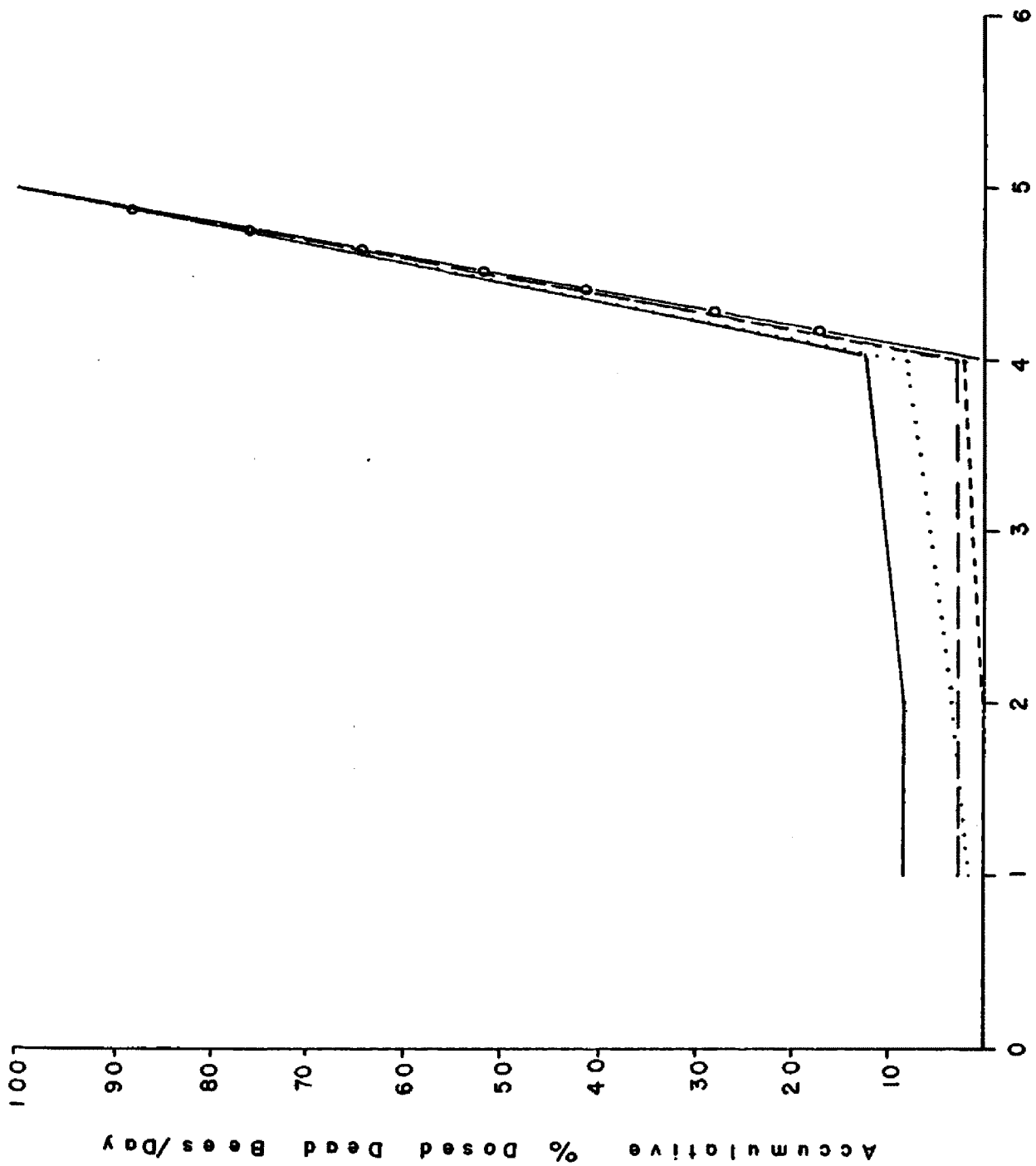


Fig. 12. Control: Tests 7-9. Theoretical Dose: 0.0 ug/bee.

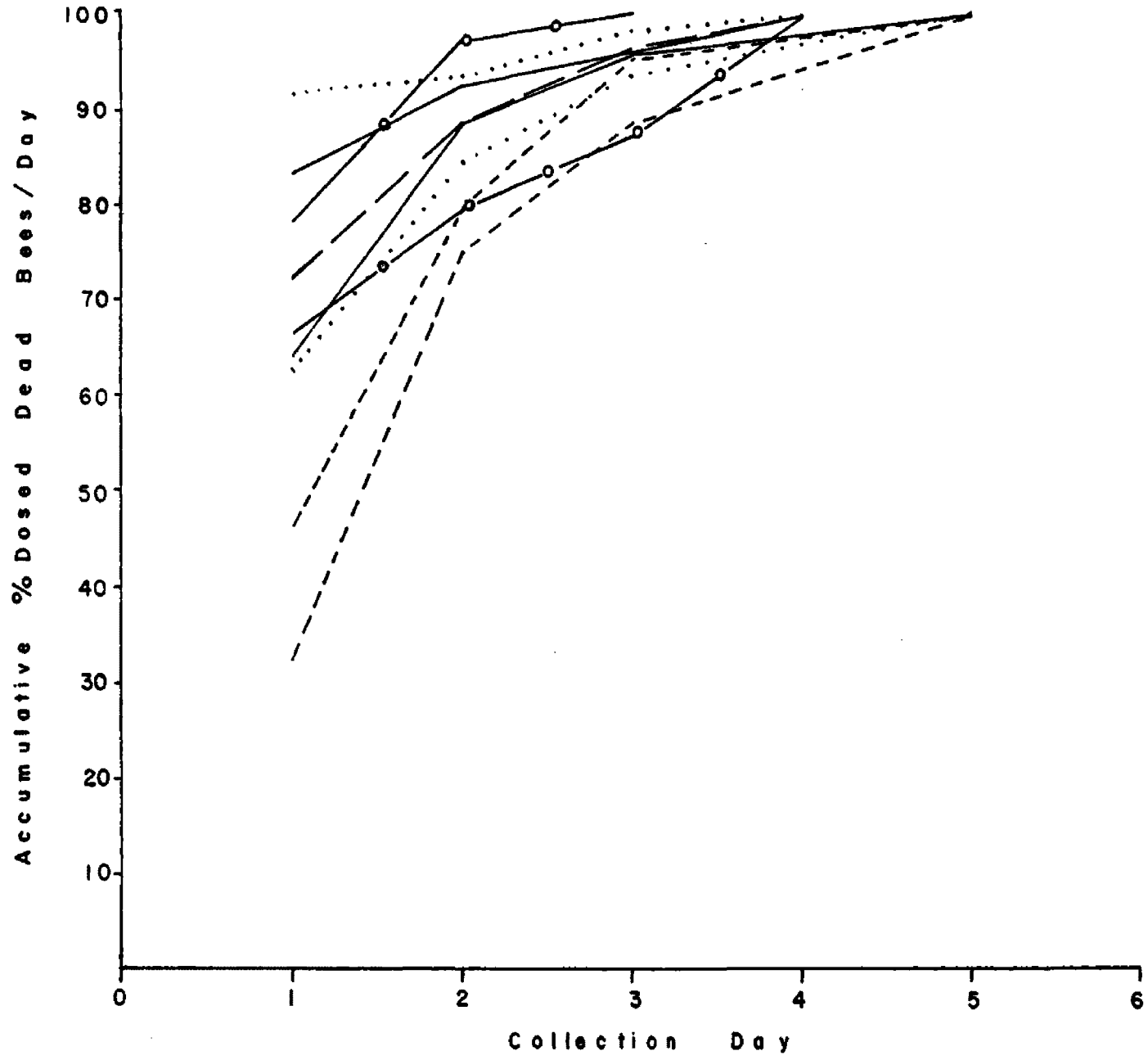


Fig. 13. Test 10 - $NaAsO_2$. Theoretical Dose: 24 hr - 10 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.65 ug/bee.

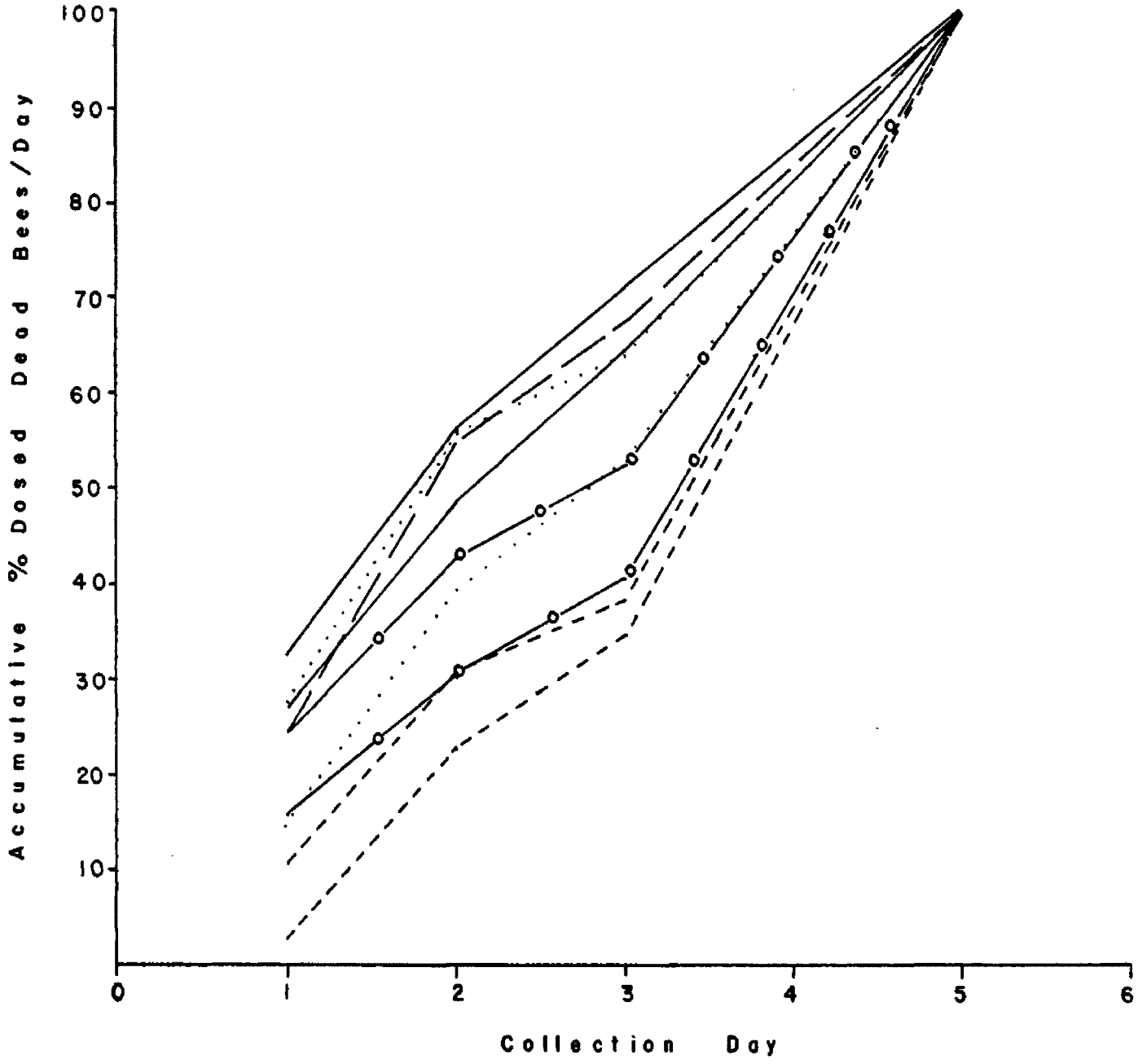


Fig. 14. Test 11 - $NaAsO_2$. Theoretical Dose: 24 hr - 5.0 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.46 ug/bee.

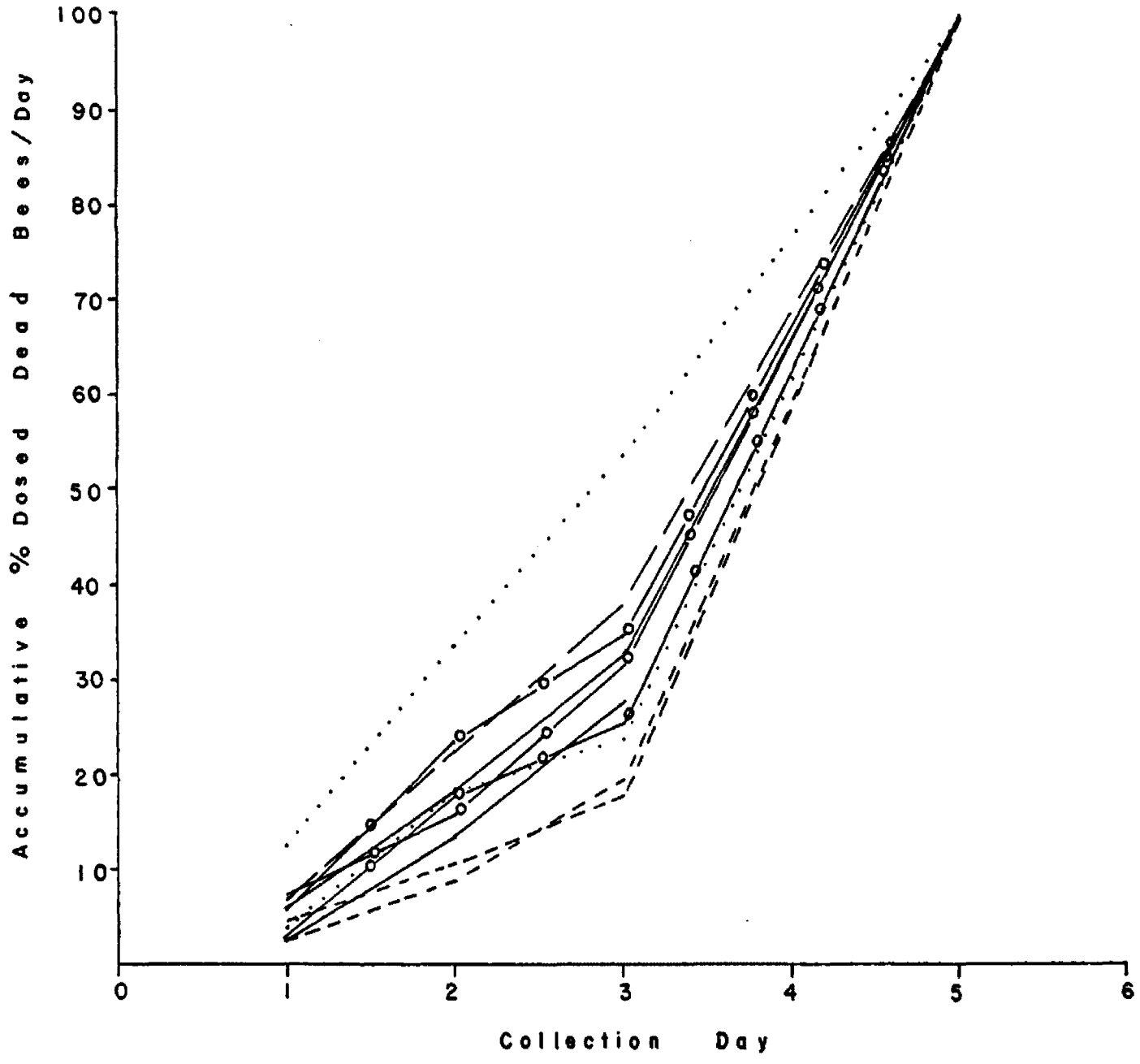


Fig. 15. Test 12 - $NaAsO_2$. Theoretical Dose: 24 hr - 3.0 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.35 ug/bee.

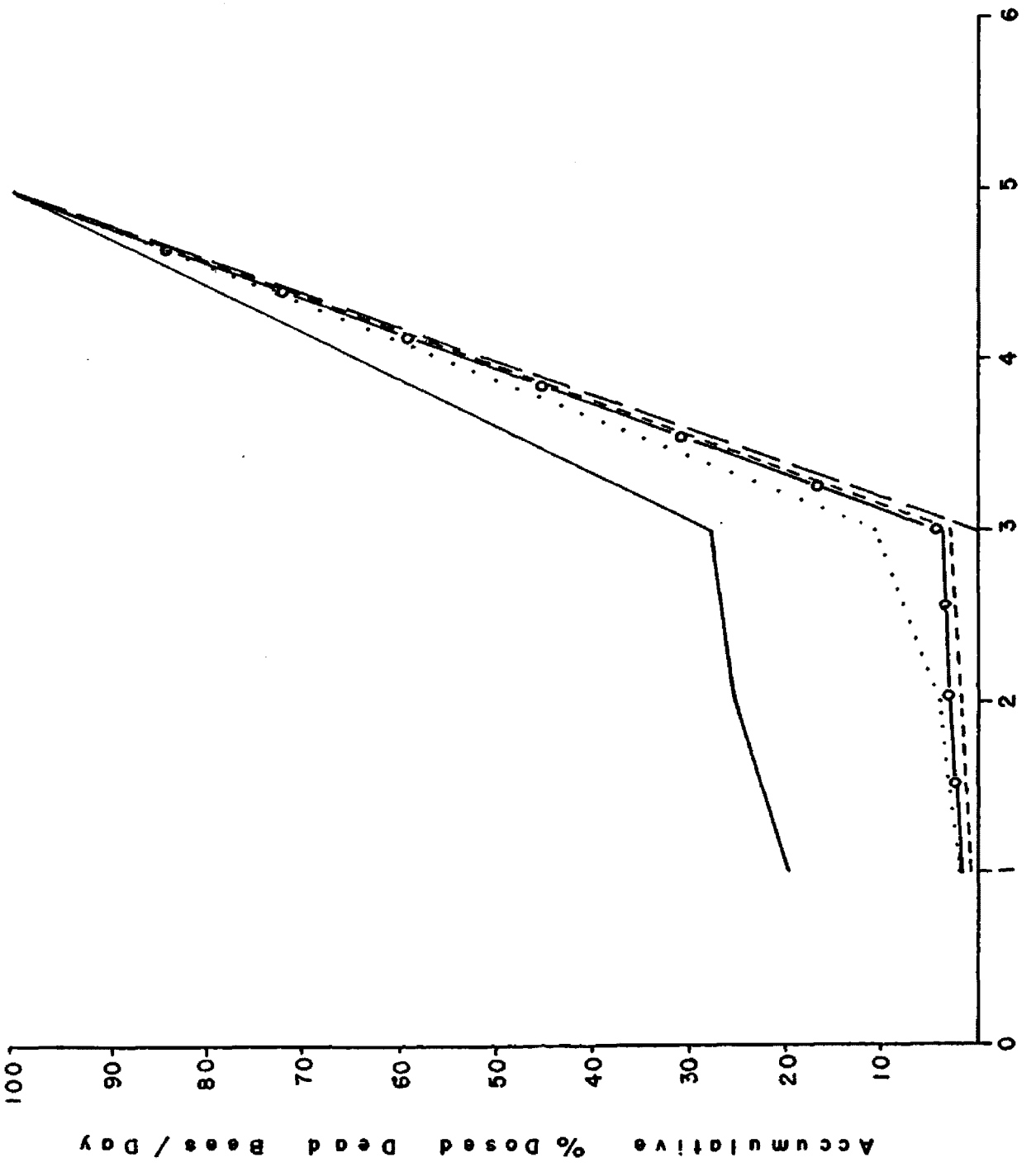


Fig. 16. Controls: Tests 10-12. Theoretical Dose: 0.0 ug/bee

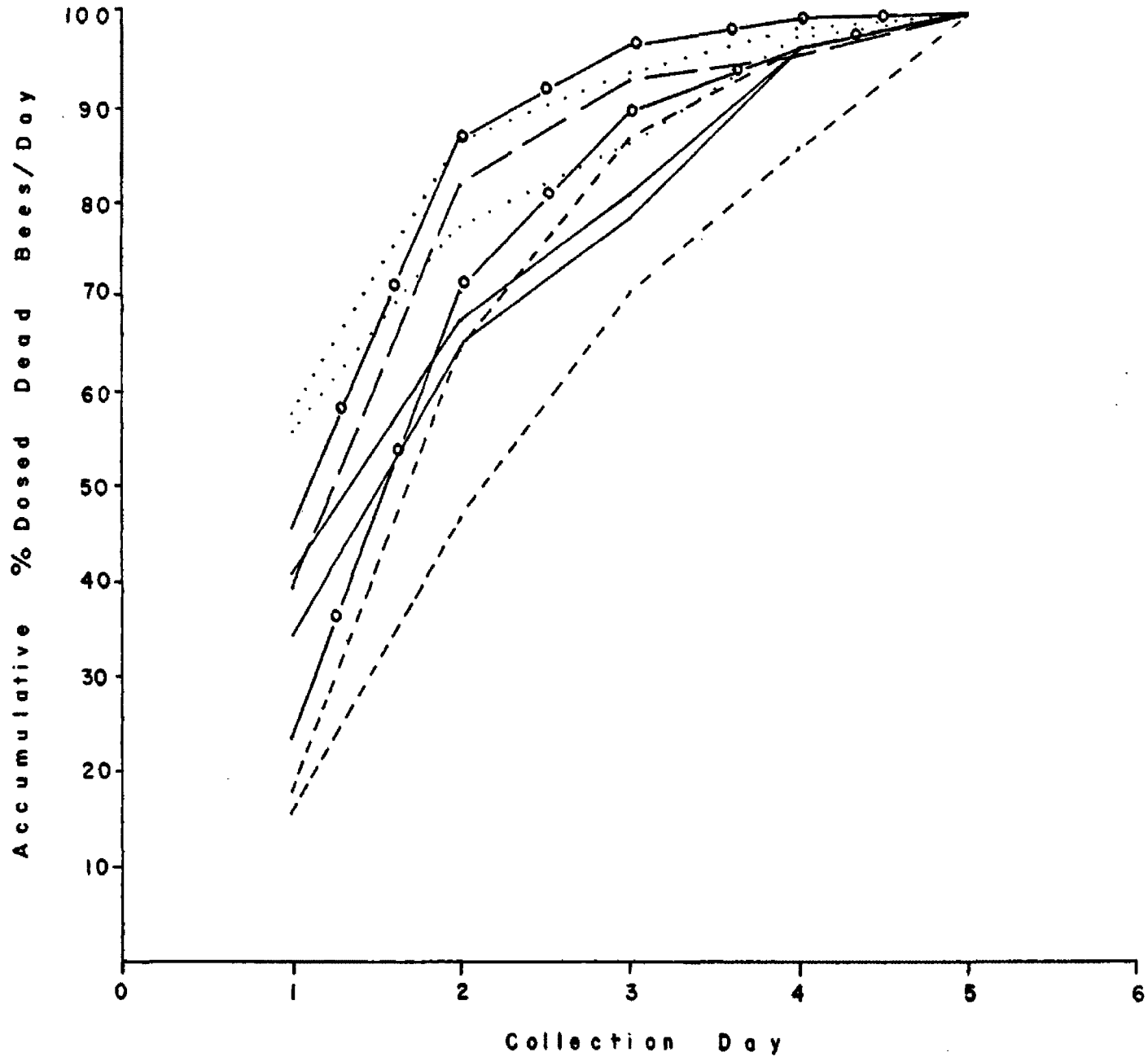


Fig. 17. Test 13 - $NaAsO_2$. Theoretical Dose: 24 hr - 8.0 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.49 ug/bee.

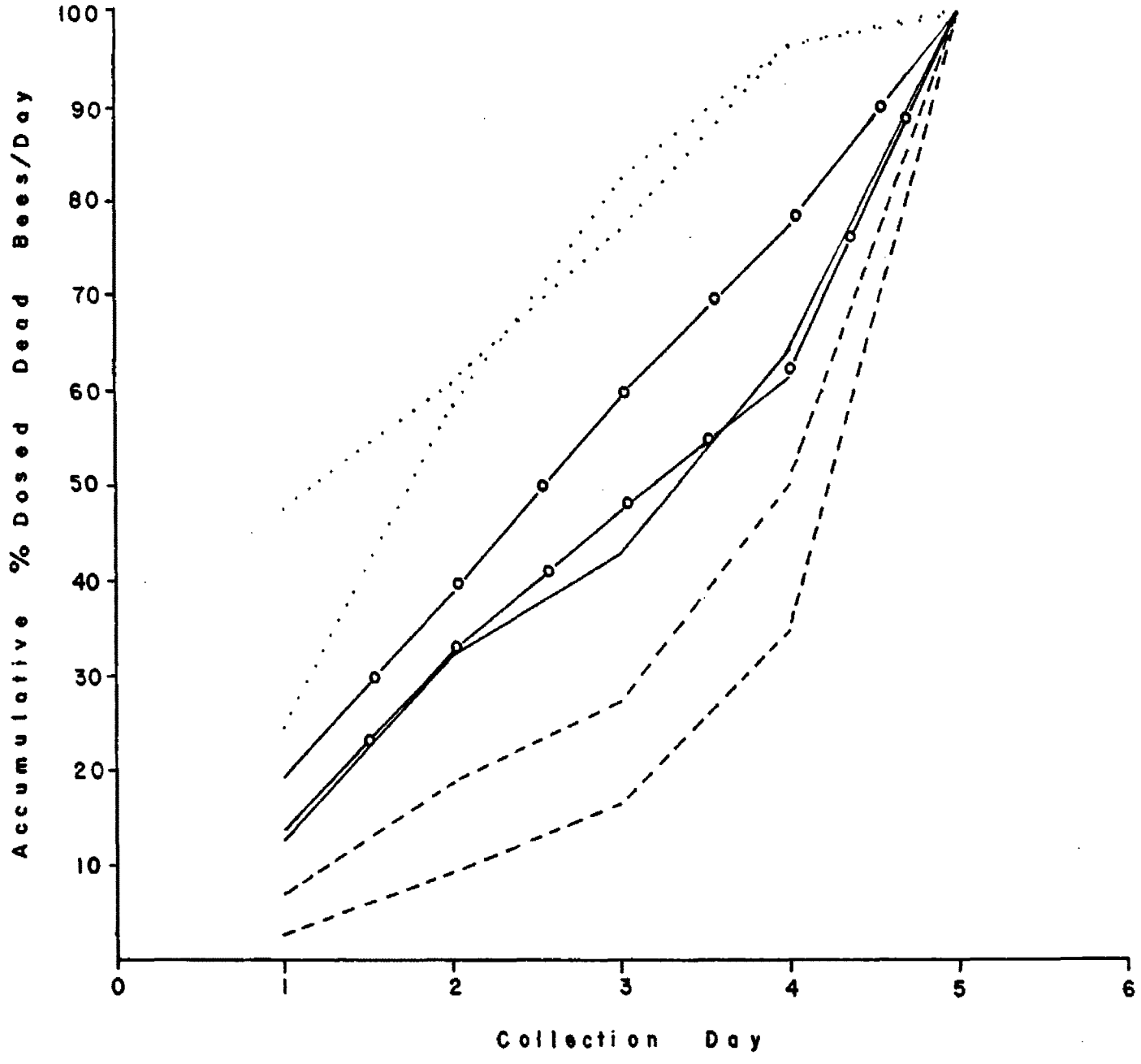


Fig. 18. Test 14 - $NaAsO_2$. Theoretical Dose: 24 hr - 7.0 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.47 ug/bee.

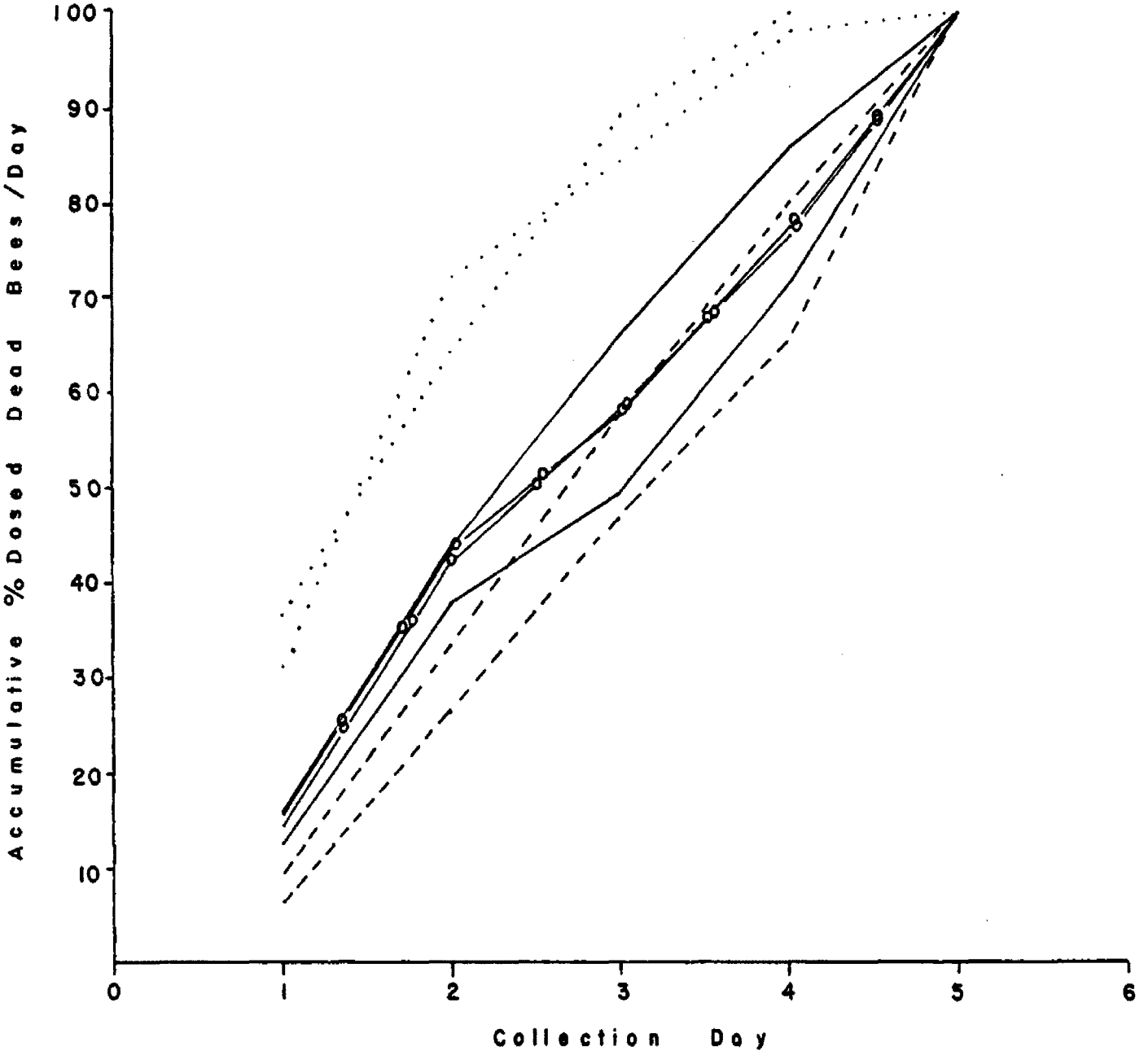


Fig. 19. Test 15 - $NaAsO_2$. Theoretical Dose: 24 hr - 6.0 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.46 ug/bee.

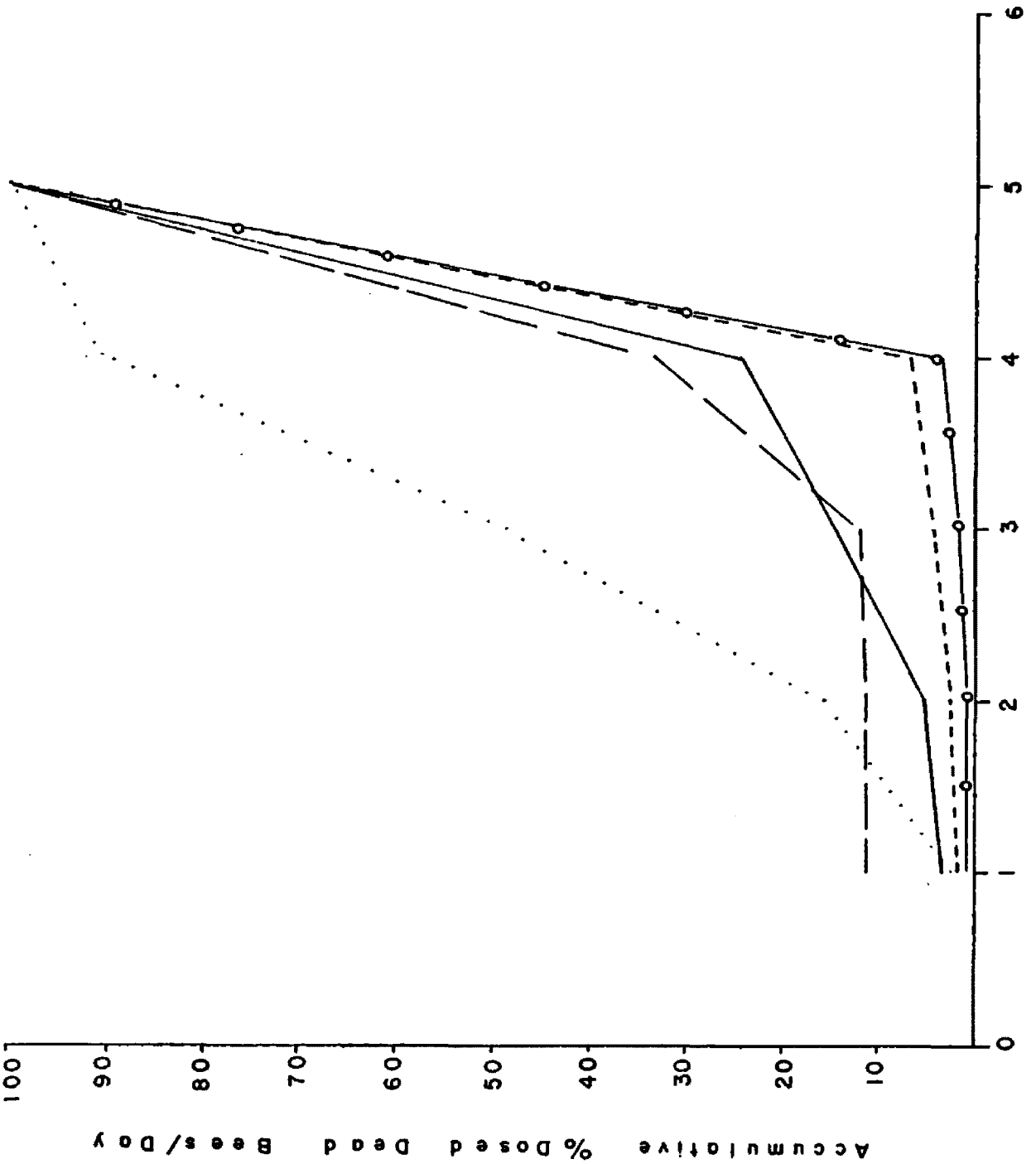


Fig. 20. Control: Tests 13-15. Theoretical Dose: 0.0 ug/bee.

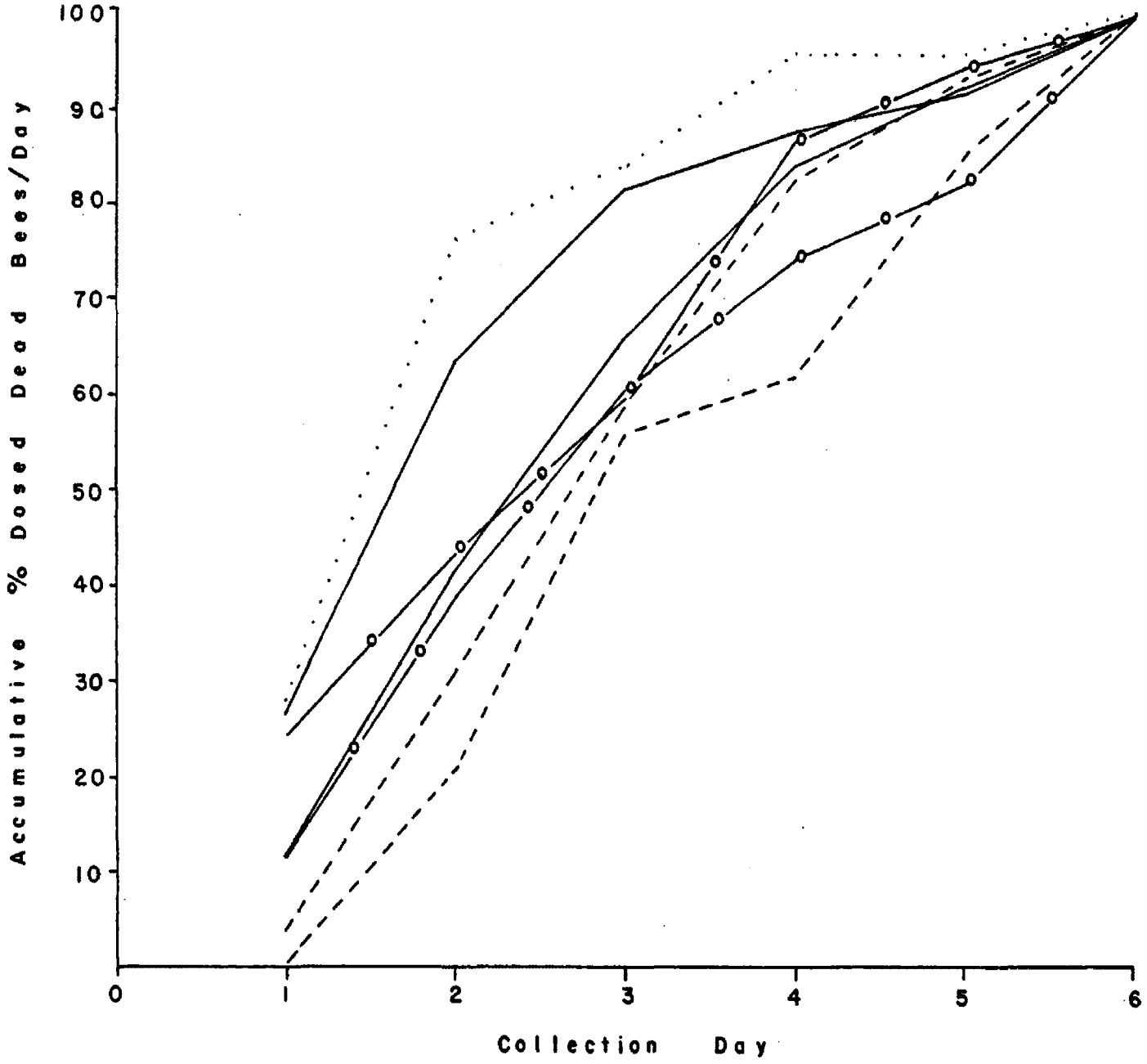


Fig. 21. Test 16 - $NaAsO_2$. Theoretical Dose: 24 hr - 4.0 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.34 ug/bee.

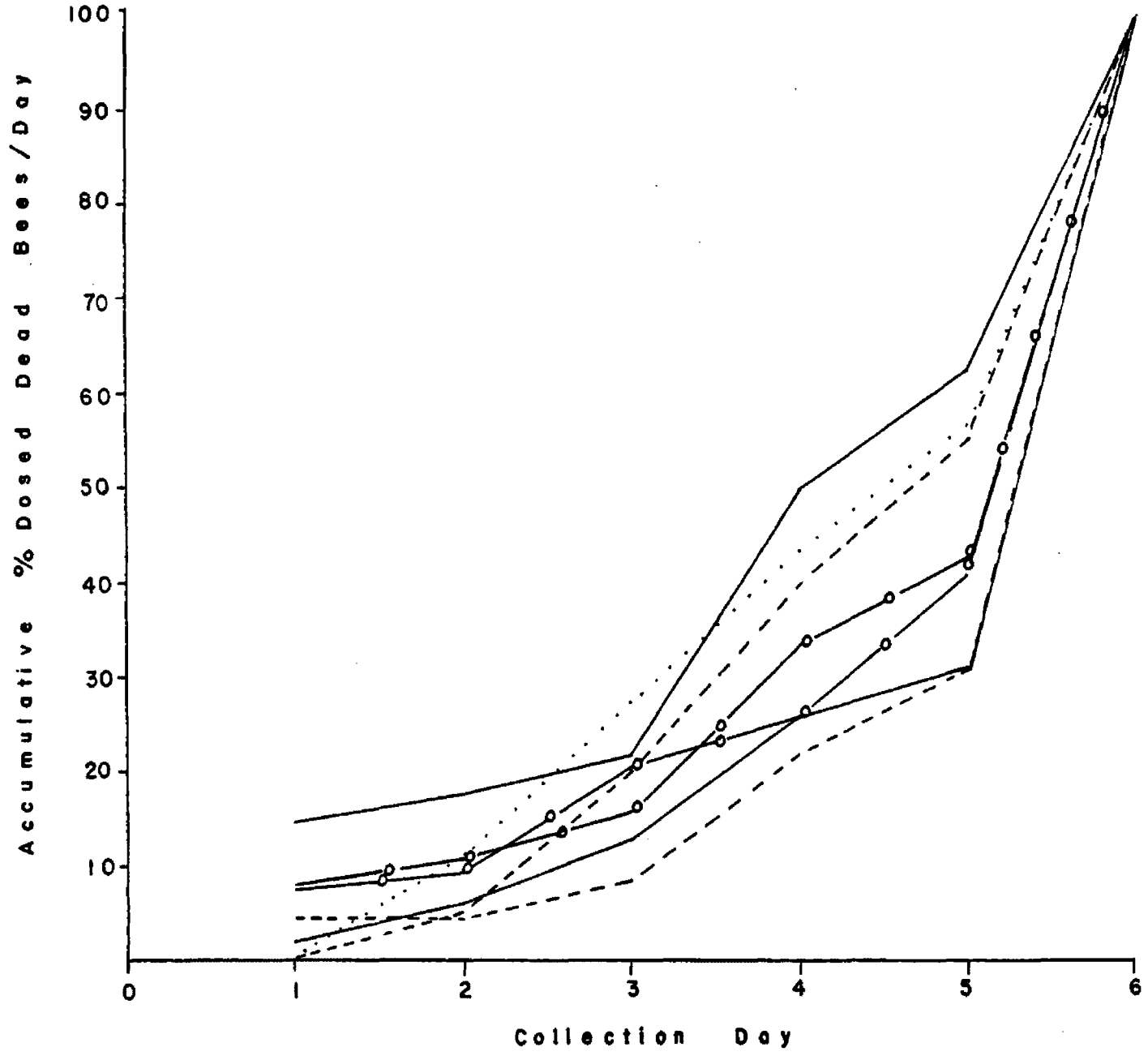


Fig. 22. Test 17 - $NaAsO_2$. Theoretical Dose: 24 hr - 2.0 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.19 ug/bee.

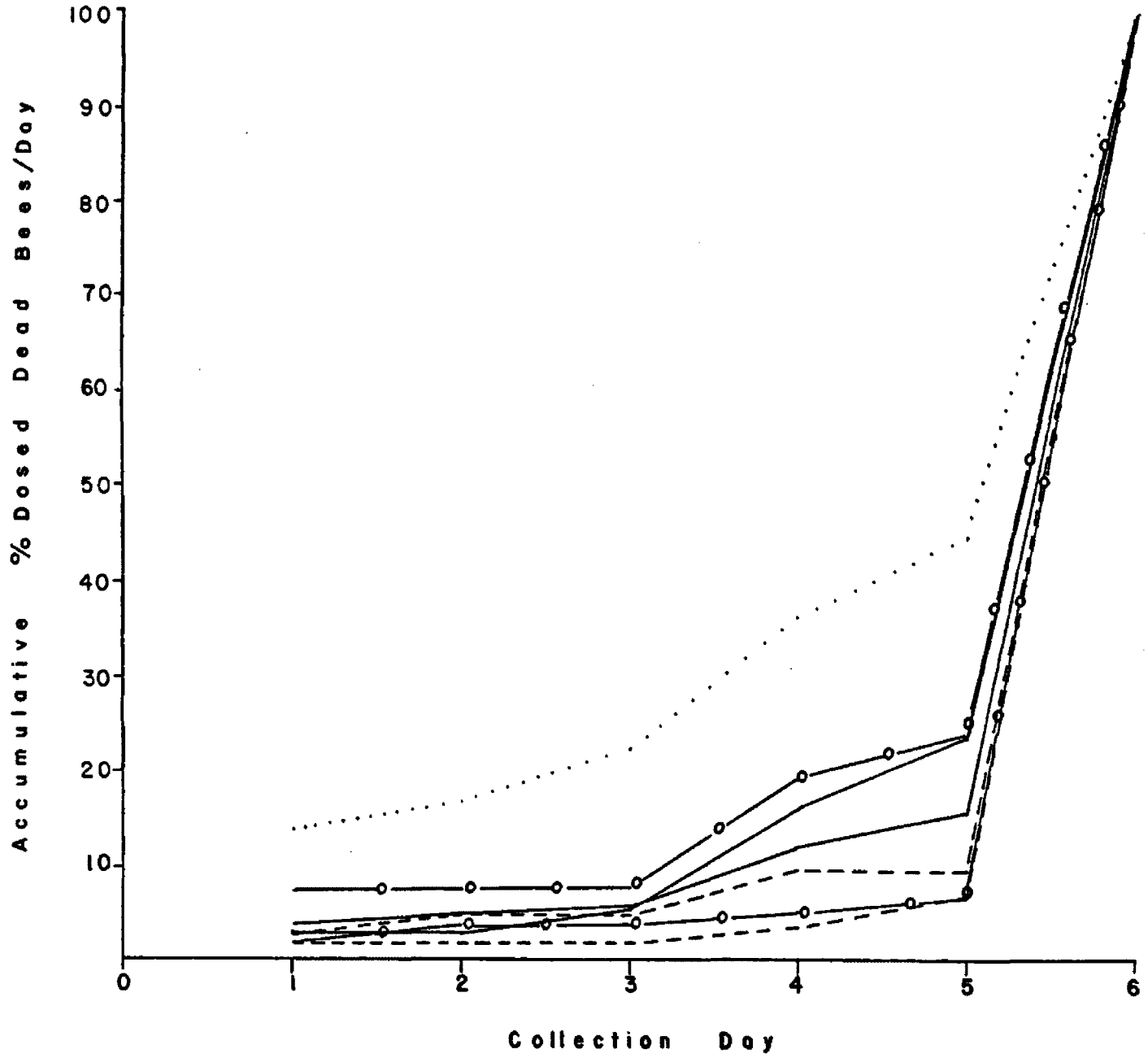


Fig. 23. Test 18 - $NaAsO_2$. Theoretical Dose: 24 hr - 1.0 ug/bee.
Actual Dose (Mean Calcconc): 24 hr - 0.12 ug/bee.

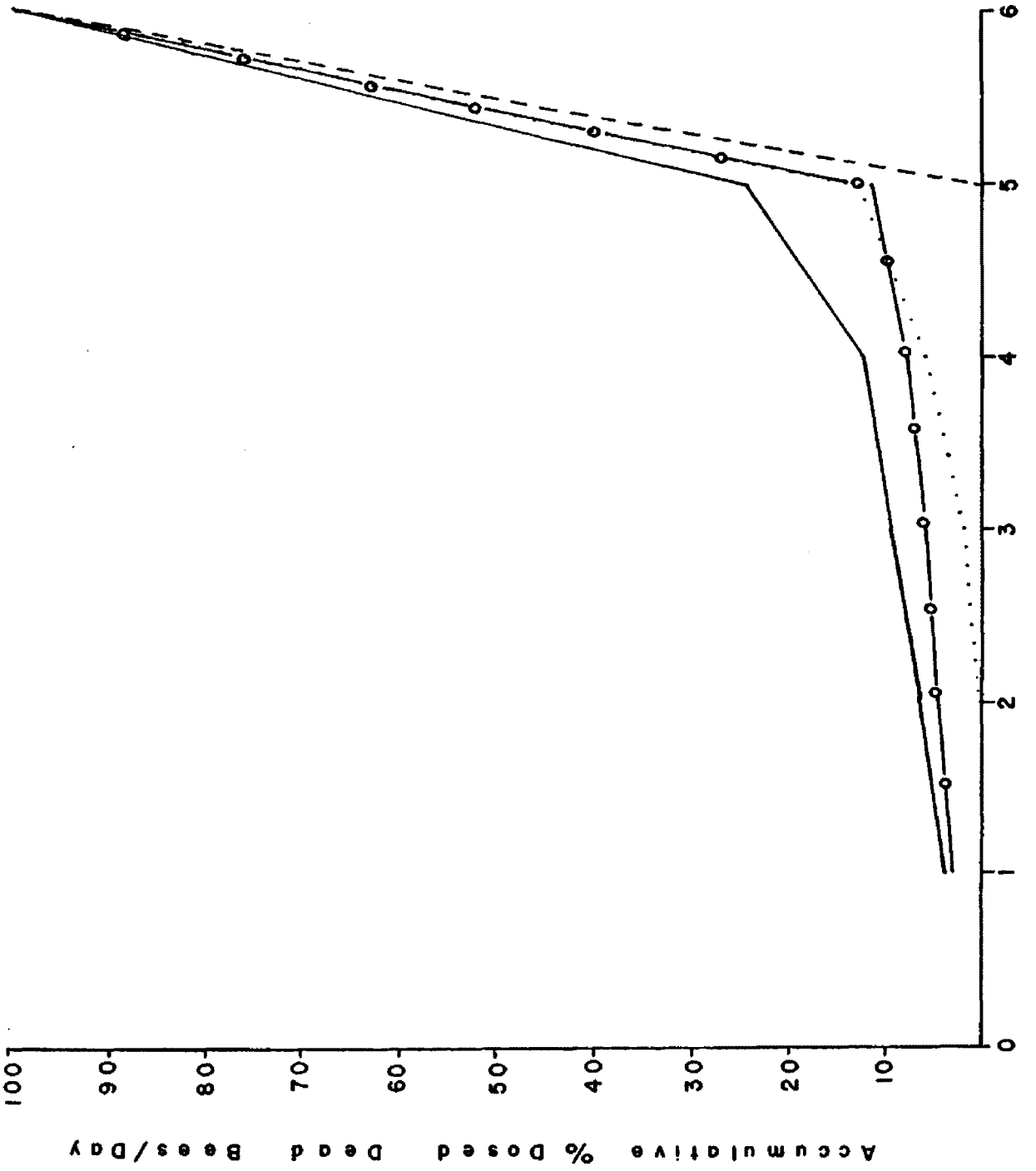


Fig. 24. Control: Tests 16-18. Theoretical Dose: 0.0 ug/bee

APPENDIX C

MEDIAN LETHAL DOSE - PROBIT ANALYSIS GRAPHS

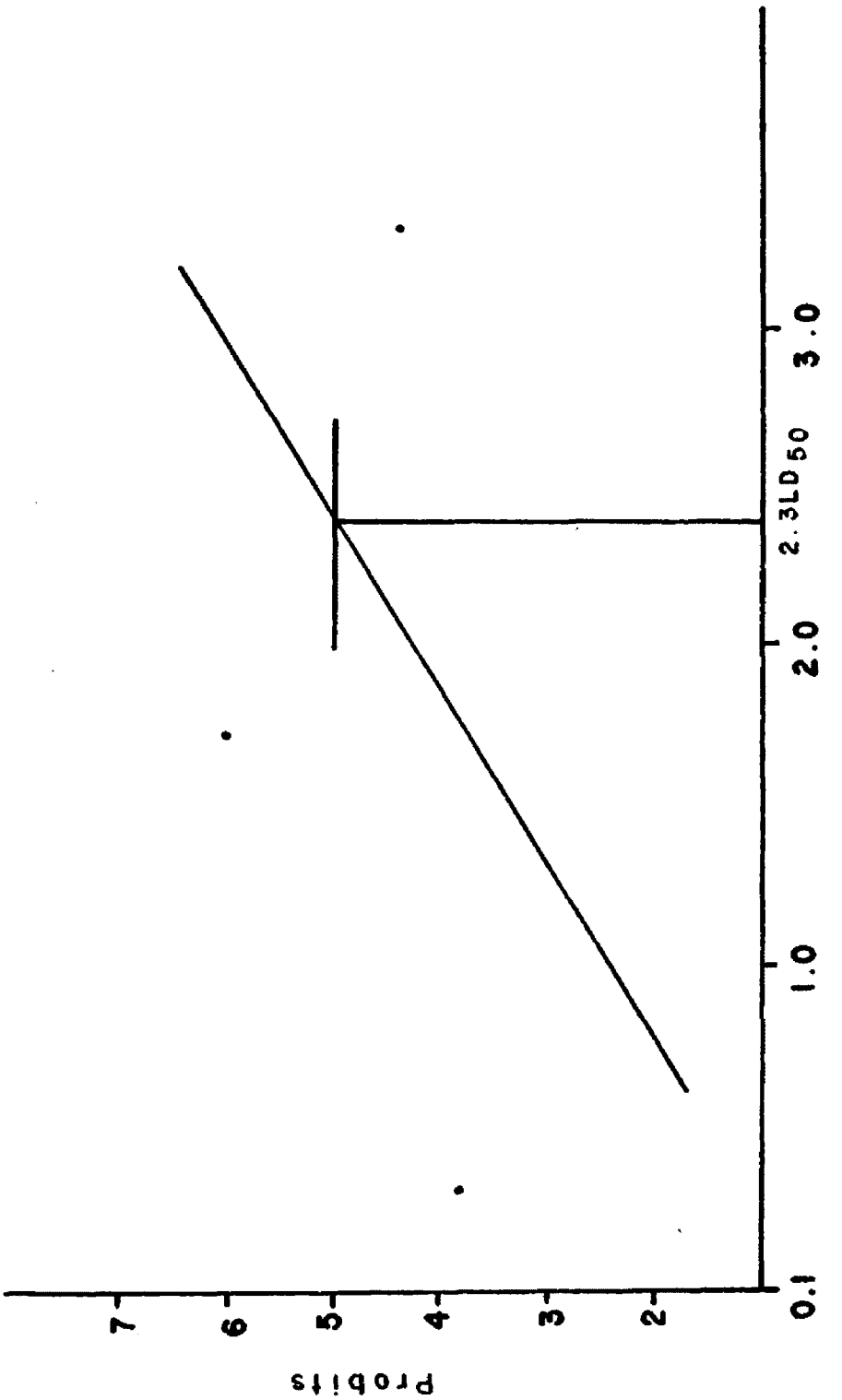


Fig. 1. As_2O_3 Pooled Data LD_{50}. Mean Dose per Bee - ug/bee (x) by Probits (y).

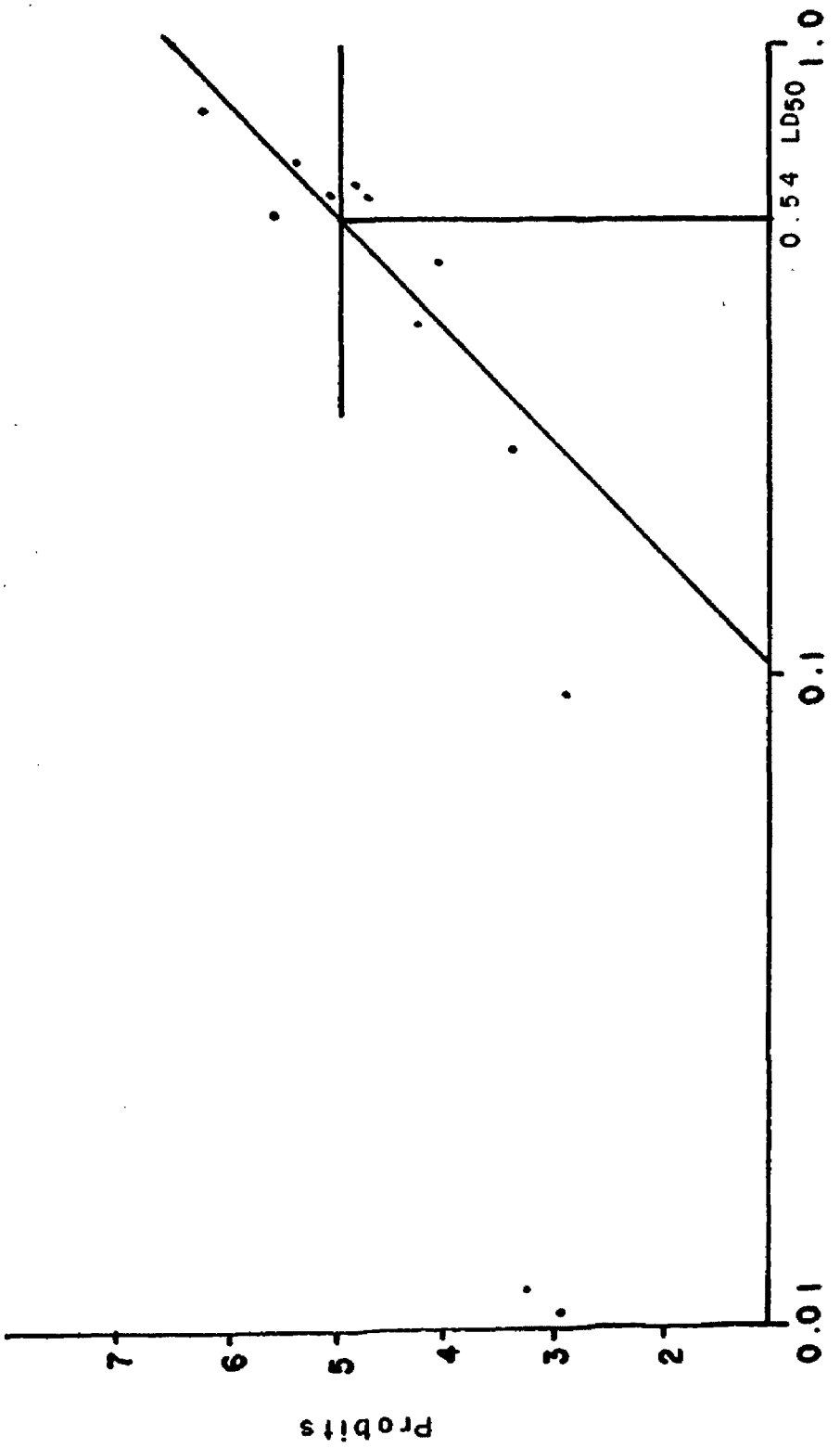


Fig. 2. $NaAsO_2$ Pooled Data LD_{50}. Mean Dose per Bee - ug/bee (x) by Probits (y).

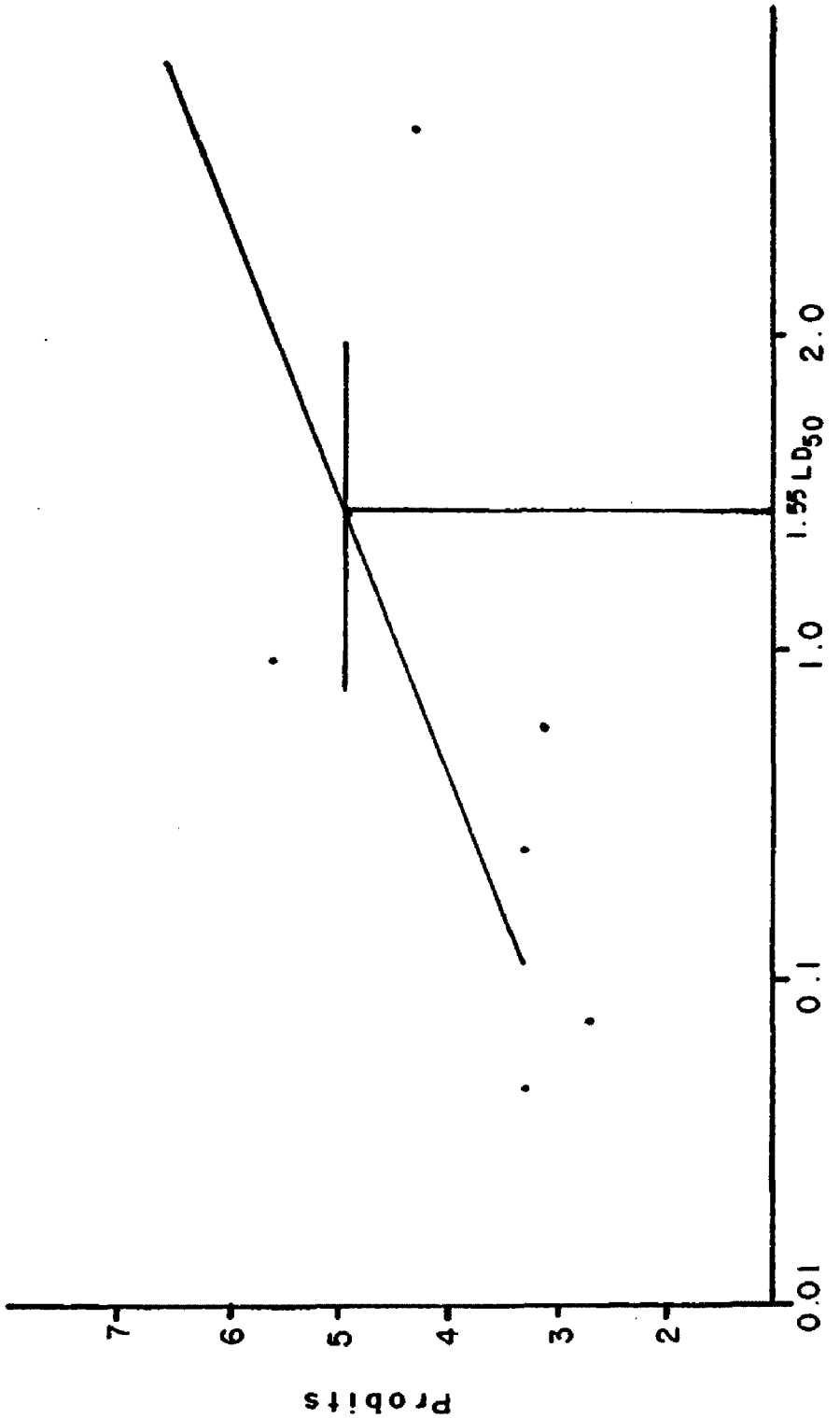


Fig. 3. As_2O_3 Colony 1 LD_{50}. Mean Dose per Bee - ug/bee (x) by Probits (y).

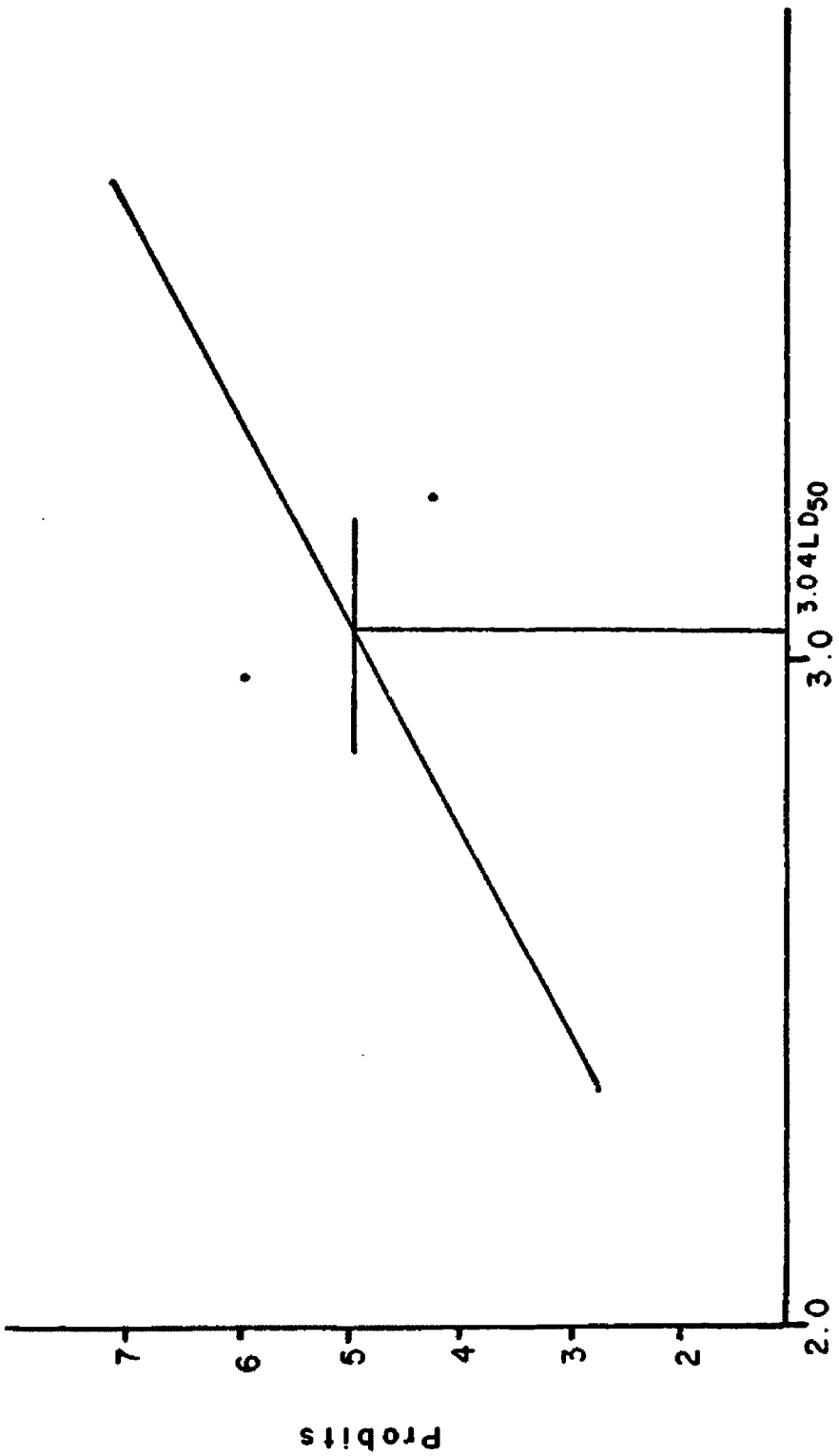


Fig. 4. As_2O_3 Colony 2 LD_{50}. Mean Dose per Bee - ug/bee (x) by Probits (y).

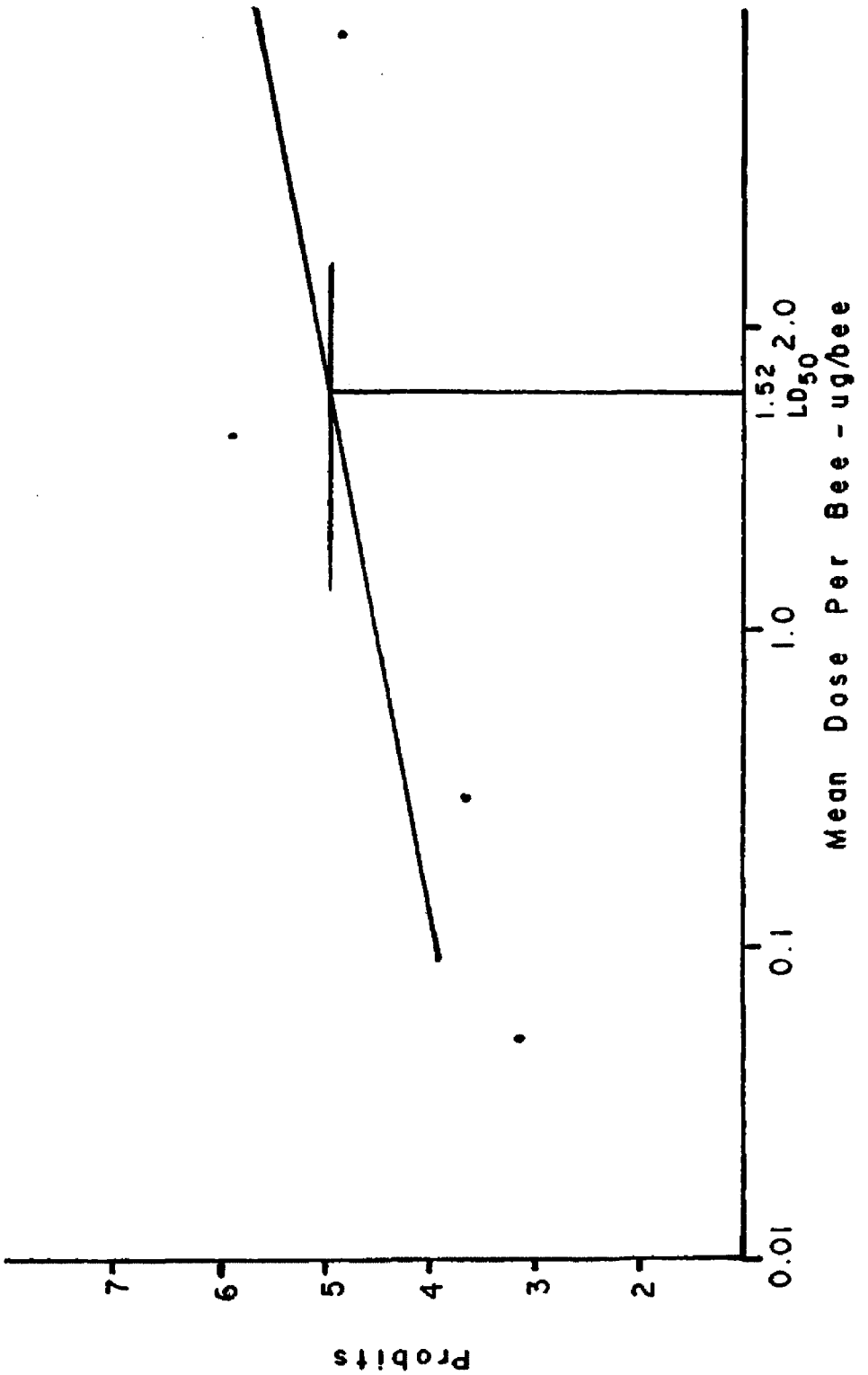


Fig. 5. As_2O_3 Colony 3 LD_{50}. Mean Dose per Bee - ug/bee (x) by Probits (y).

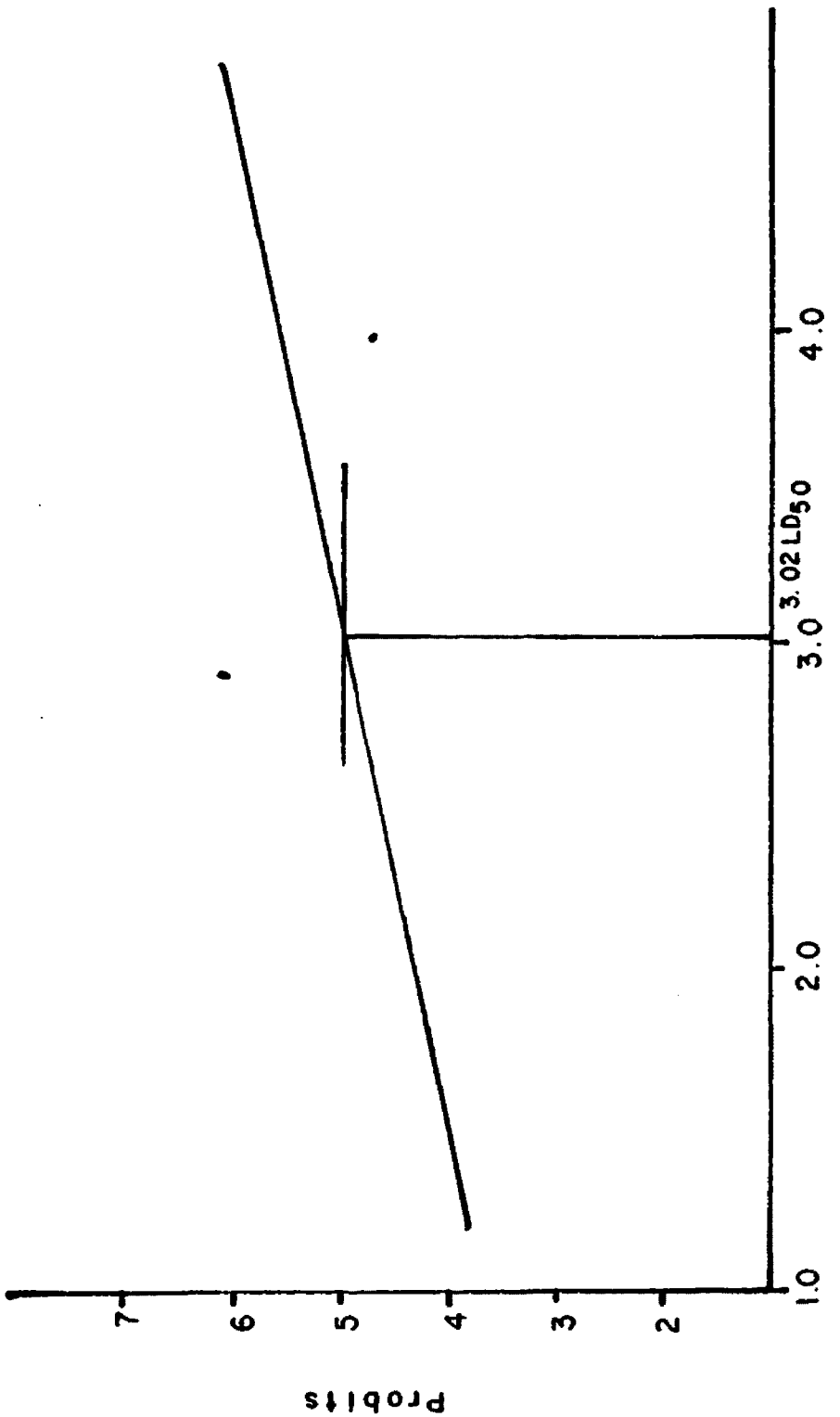


Fig. 6. As_2O_3 Colony 4 LD_{50}. Mean Dose per Bee - ug/bee (x) by Probits (y).

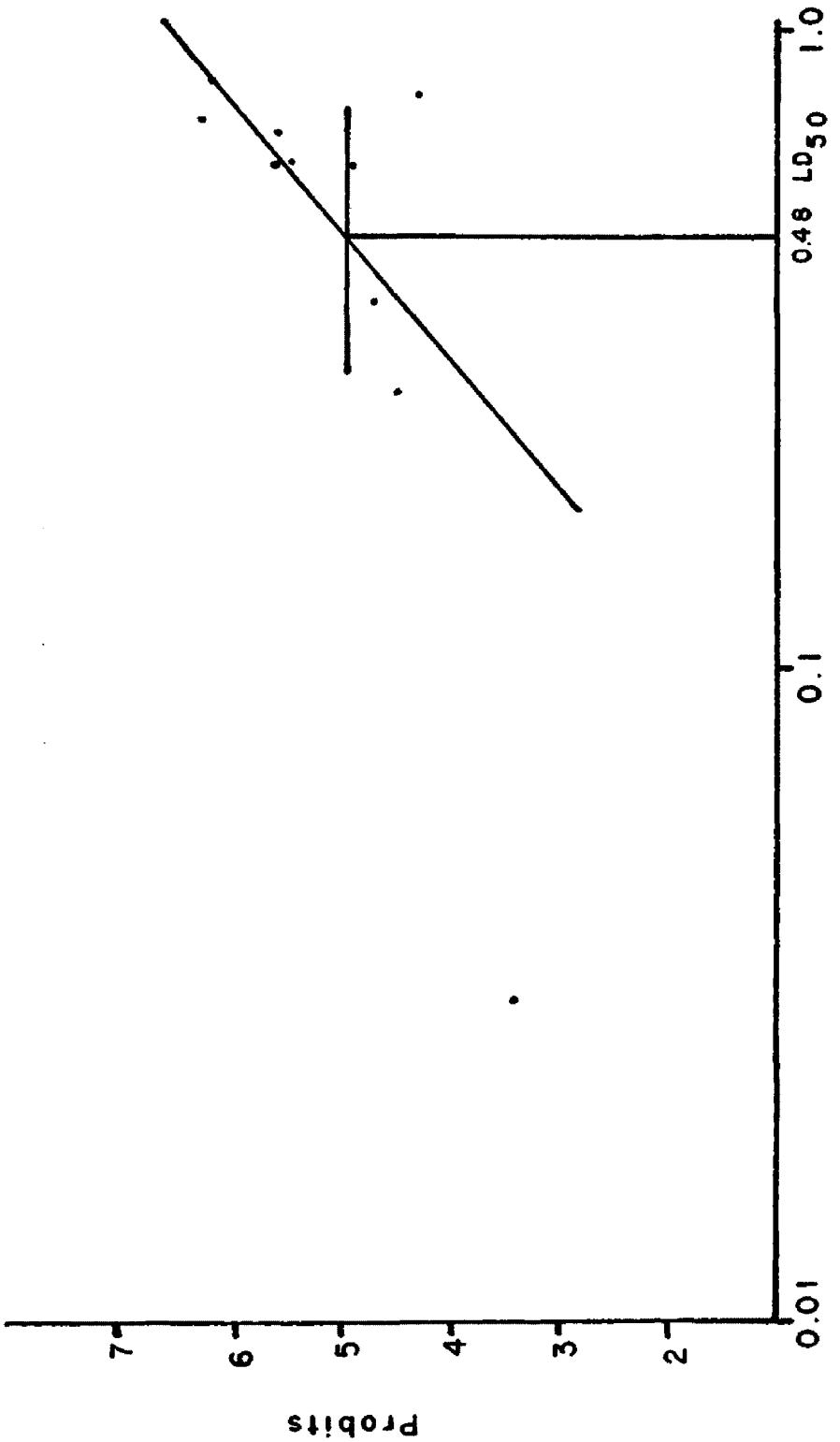


Fig. 7. $NaAsO_2$ Colony 1 LD_{50}. Mean Dose per Bee - ug/bee (x) by Probits (y).

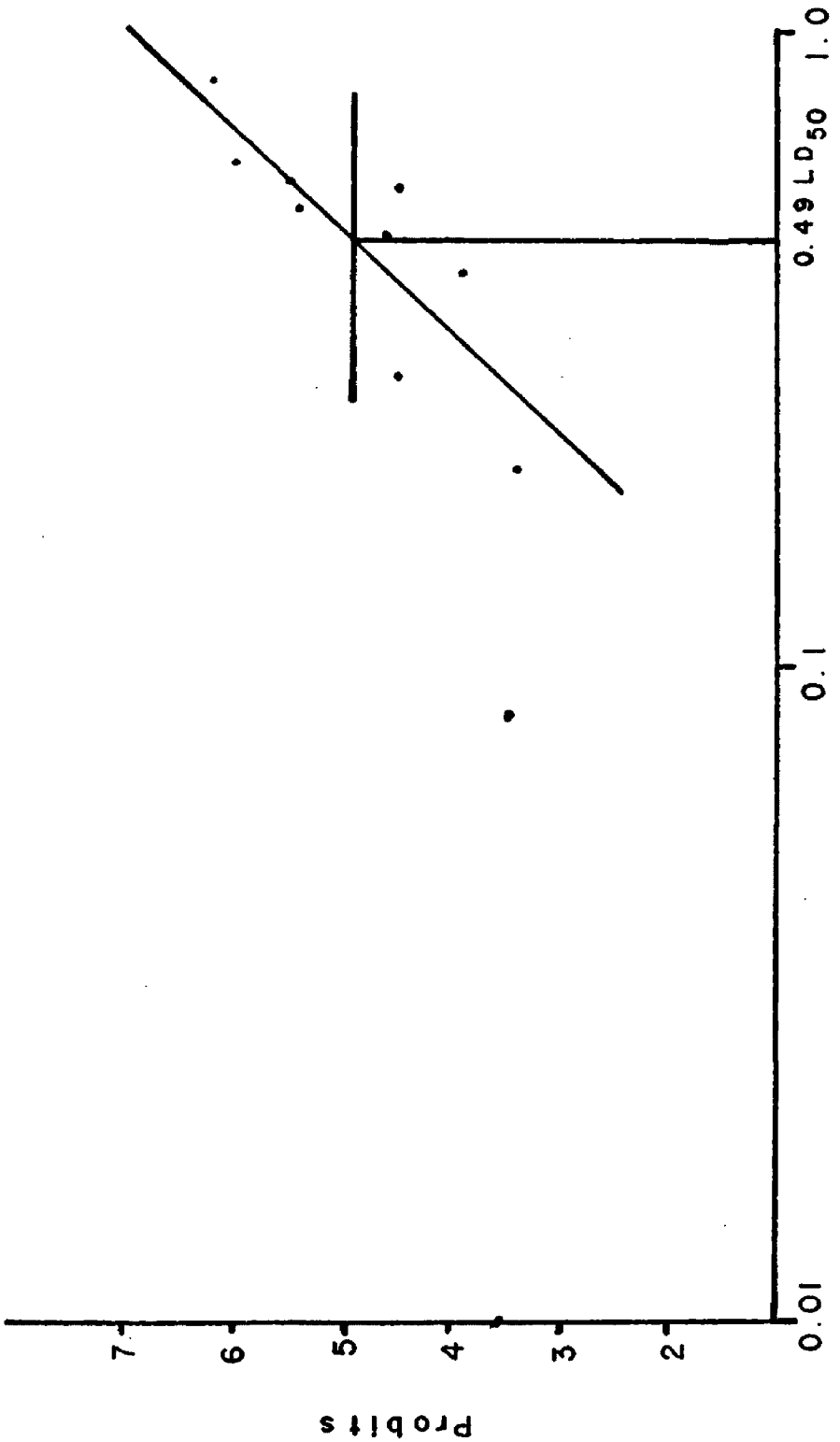


Fig. 8. $NaAsO_2$ Colony 2 LD_{50}. Mean Dose per Bee - ug/bee (x) by Probits (y).

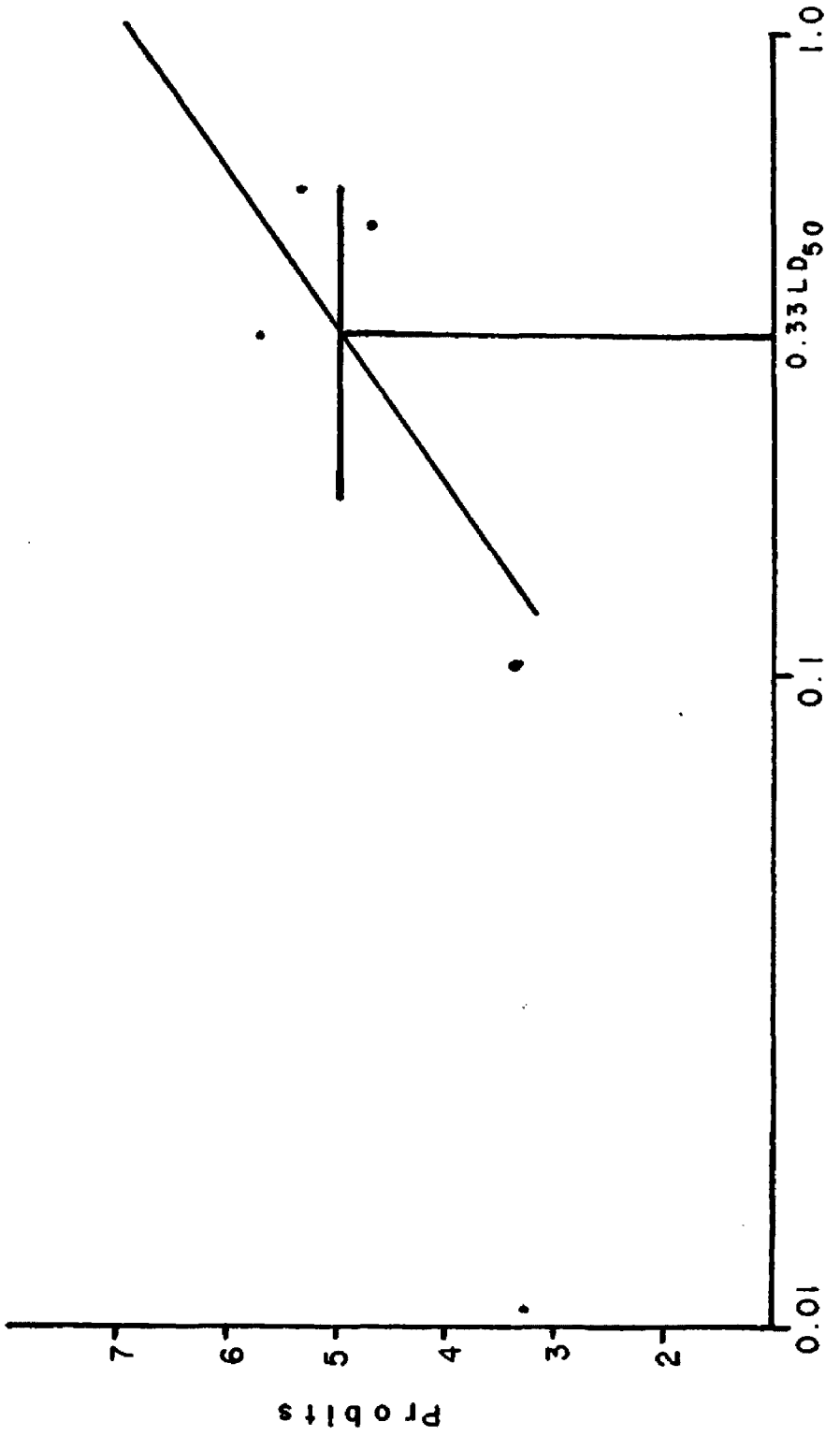


Fig. 9. $NaAsO_2$ Colony 3 LD_{50}. Mean Dose per Bee - ug/bee (x) by Probits (y).

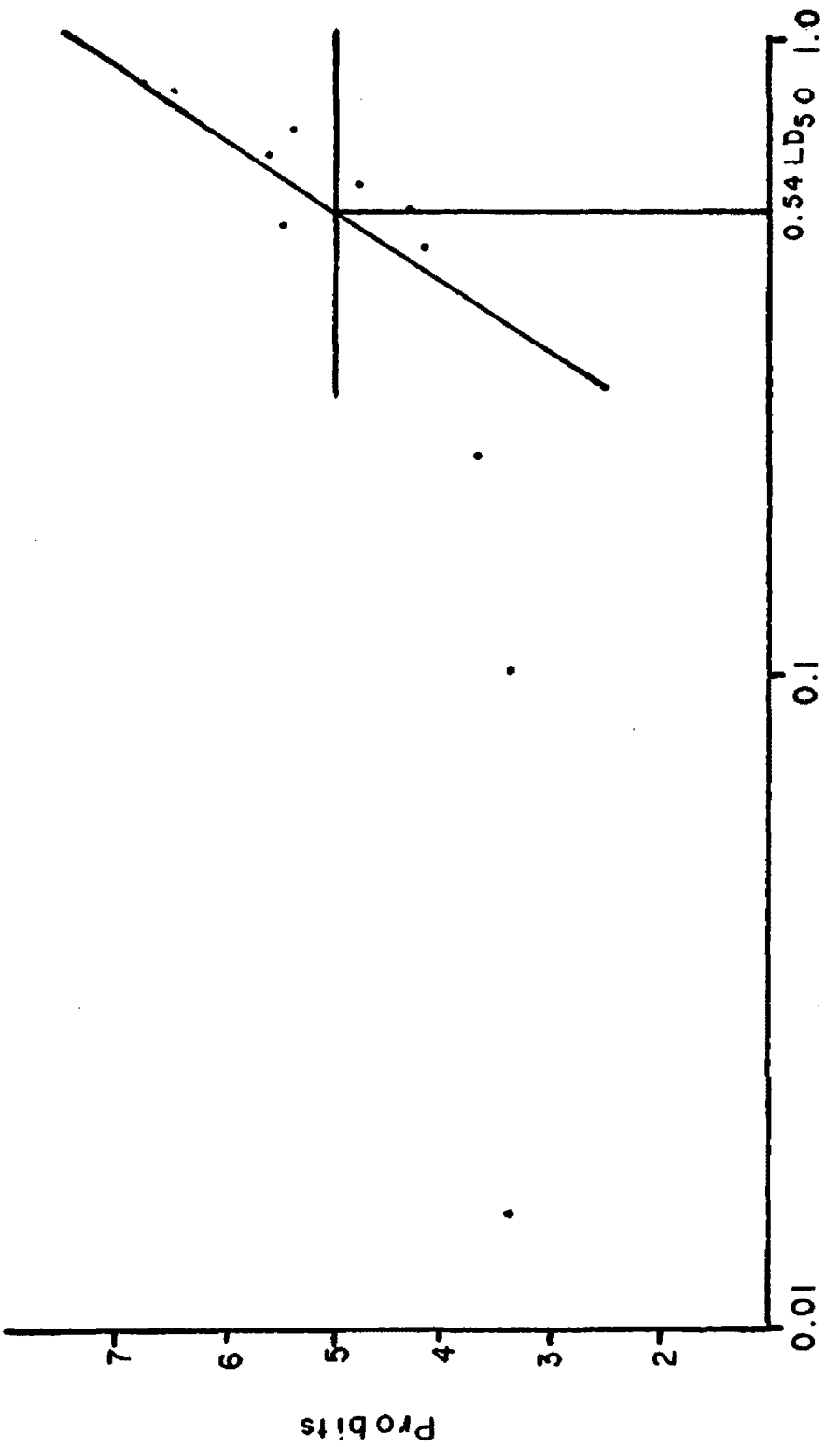


Fig. 10. $NaAsO_2$ Colony 4 LD_{50}. Mean Dose per Bee - ug/bee (x) by Probits (y).

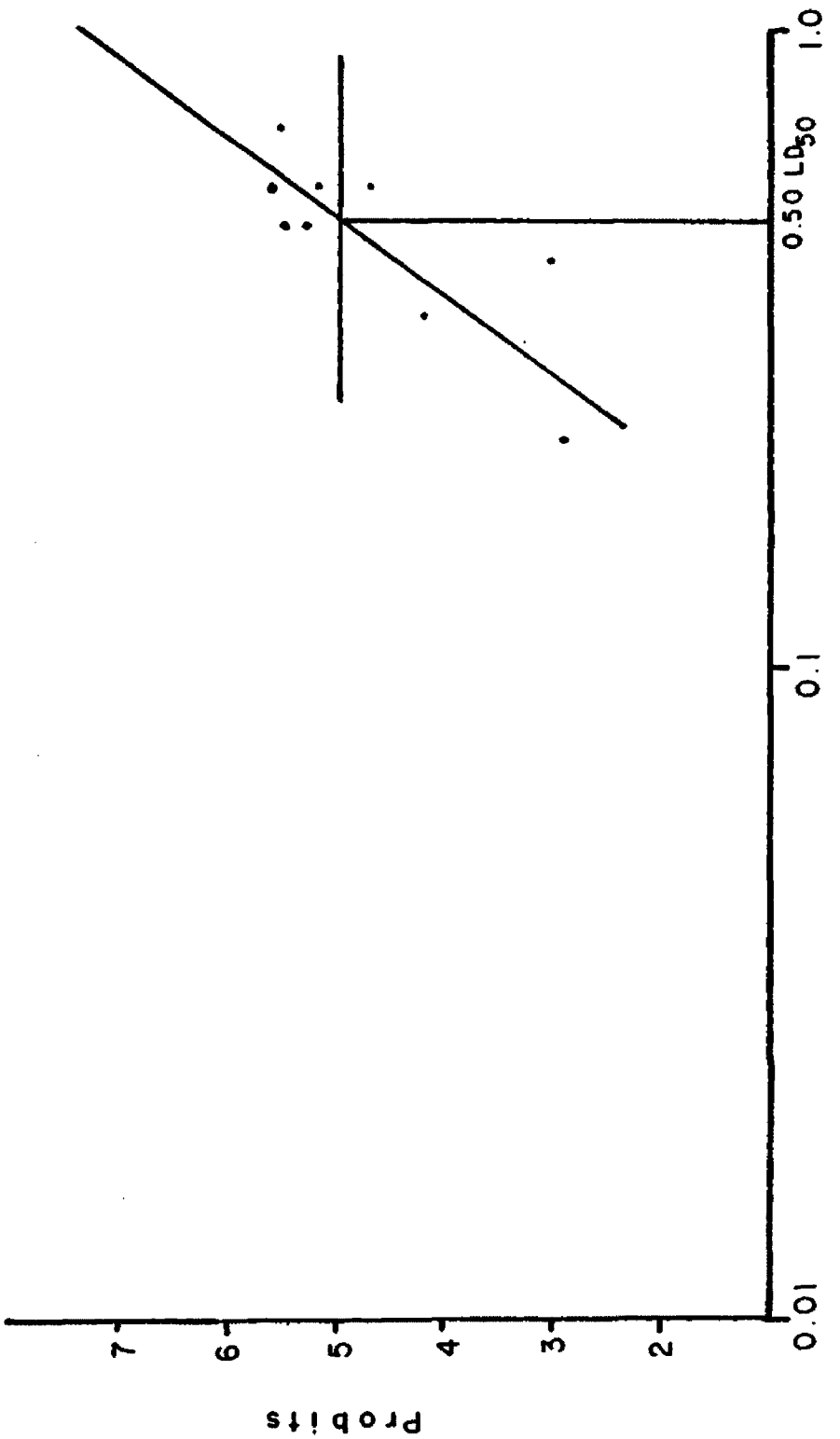


Fig. 11. $NaAsO_2$ Colony 5 LD_{50}. Mean Dose per Bee - ug/bee (x) by Probits (y).